

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
25 March 2004 (25.03.2004)

PCT

(10) International Publication Number
WO 2004/024183 A1

- (51) International Patent Classification⁷: **A61K 39/39**, 39/385, 39/00, C07K 14/435, A61P 3/04
- (21) International Application Number:
PCT/DK2003/000592
- (22) International Filing Date:
12 September 2003 (12.09.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
PA 2002 01345 12 September 2002 (12.09.2002) DK
60/410,164 12 September 2002 (12.09.2002) US
- (71) Applicant (*for all designated States except US*):
PHARMEXA A/S [DK/DK]; Kogle Allé 6, DK-2970
Hørsholm (DK).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): BØVING, Tine,
Elisabeth, Gottschalk [DK/DK]; c/o PHARMEXA A/S,
Kogle Allé 6, DK-2970 Hørsholm (DK). KLYSNER,
Steen [DK/DK]; c/o PHARMEXA A/S, Kogle Allé 6,
DK-2970 Hørsholm (DK).
- (74) Agent: INSPICOS A/S; Bøge Allé 3, P.O. Box 45,
DK-2970 Hørsholm (DK).
- (81) Designated States (*national*): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, EG, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK (utility model), SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
- with international search report
 - before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: IMMUNIZATION AGAINST AUTOLOGOUS GHRELIN

(57) Abstract: Disclosed are novel methods that generally rely on immunization against autologous ghrelin. Immunization is preferably effected by administration of analogues of autologous ghrelin, said analogues being capable of inducing antibody production against the autologous ghrelin polypeptides. Especially preferred as an immunogen is autologous ghrelin, which has been modified by introduction of one single or a few foreign, immunodominant and promiscuous T-cell epitopes. Also disclosed are nucleic acid vaccination against ghrelin and vaccination using live vaccines as well as methods and means useful for the vaccination. Such methods and means include methods for the preparation of analogues and pharmaceutical formulations, as well as nucleic acid fragments, vectors, transformed cells, polypeptides and pharmaceutical formulations.

WO 2004/024183 A1

IMMUNIZATION AGAINST AUTOLOGOUS GHRELIN

FIELD OF THE INVENTION

The present invention relates to therapeutic vaccination ("active therapeutic immunotherapy"). In particular it relates to therapeutic vaccination that targets autologous ("self") ghrelin protein and therapy targeting obesity and other diseases characterized by excess body fat deposits or, alternatively, conditions where an increase in body weight are of interest.

The present invention thus relates to improvements in therapy and prevention of obesity characterized by excess fat deposition, but also to improvements in therapy and prevention of conditions characterized in loss of body weight. More specifically, the present invention provides a method for down-regulating (undesired) deposits of fat by enabling the production of antibodies against ghrelin or components thereof in subjects suffering from or in danger of suffering from obesity involving excess fat deposition. The invention further provides a method for up-regulating desired deposits of body fat by enabling the production of antibodies against ghrelin or components thereof in subjects suffering from or in danger of suffering from emaciation. The invention also provides for methods of producing polypeptides useful in these methods as well as for the modified polypeptides as such. Also encompassed by the present invention are nucleic acid fragments encoding the modified polypeptides as well as vectors incorporating these nucleic acid fragments and host cells and cell lines transformed therewith. The invention also provides for a method for the identification of analogues of the deposit polypeptides which are useful in the methods of the invention as well as for compositions comprising modified polypeptides or comprising nucleic acids encoding modified polypeptides. Finally, the present invention also provides for conjugate ghrelin peptide immunogens.

BACKGROUND OF THE INVENTION

Over the past three decades, the prevalence of obesity has risen to reach epidemic proportions not only in the United States and Europe but also developing countries like China, Latin American-, Middle-East- and North African countries are now reporting increasing incidences of obesity amongst the populations. Despite public health efforts, no marked shift towards healthier lifestyles is likely over the next ten years. According to recent statistics, results indicate that an estimated 61% of U.S. adults are either overweight or obese, defined as having a body mass index ($BMI = \text{Weight in kilograms} \div [\text{Height in meters}]^2$) of 25 or more (National Health and Nutrition Examination Survey (NHANES) 1999). In the same population, obesity

(defined as BMI greater than or equal to 30.0) has nearly doubled from approximately 15% in 1980 to an estimated 27% in 1999. It is, according to the WHO, estimated that globally 300 mill people are obese.

Overweight and obese individuals (BMI of 25 and above) are at increased risk for physical ailments such as: High blood pressure, hypertension; High blood cholesterol, dyslipidemia; Type 2 (non-insulin dependent) Diabetes; Insulin resistance, glucose intolerance; Hyperinsulinemia; Coronary heart disease; Angina pectoris; Congestive heart failure; Stroke; Gallstones; Cholecystitis and cholelithiasis; Gout; Osteoarthritis; Obstructive sleep apnoea and respiratory problems; Some types of cancer (such as endometrial, breast, prostate, and colon); Complications of pregnancy; Poor female reproductive health (such as menstrual irregularities, infertility, irregular ovulation); Bladder control problems (such as stress incontinence); Uric acid nephrolithiasis; Psychological disorders (such as depression, eating disorders, distorted body image, and low self esteem). Its health consequences range from increased risk of premature death to serious chronic conditions that reduce the overall quality of life. Furthermore, severe obesity is associated with a 12 fold increase in mortality in 25-35 year olds when compared to lean individuals. Negative attitudes towards the obese can lead to discrimination in many areas of their life including health care and employment.

The direct cost of diagnosis, treatment and management of obesity within national health systems has only been assessed in a few countries to date. Although the methodology varied considerably between studies, making it difficult to compare costs across countries and to extrapolate the results from one country to another, these estimates suggest that between 2-8% of the total sick care costs in Western countries are attributable to obesity. This represents a major fraction of national health care budgets comparable with for example, the total cost of cancer therapy. The potential impact on health care resources in the less developed health care systems of developing countries is likely to be even more severe (WHO).

Overweight and obesity result from an imbalance involving excessive calorie consumption and/or inadequate physical activity. For each individual, body weight is the result of a combination of genetic, metabolic, behavioural, environmental, cultural, and socio-economic influences. Behavioural and environmental factors are large contributors to overweight and obesity and provide the greatest opportunity for actions and interventions designed for prevention and treatment. Hence, many studies have demonstrated that reduction in obesity by diet and exercise reduces the risk factors mentioned above dramatically. Unfortunately, these treatments are largely unsuccessful with a failure rate reaching 95%. This failure may be due to a complex body mechanism, which in ancient times helped us survive when food supplies were unreliable. The mechanism may contribute to increased appetite, preference for highly caloric foods, reduced physical activity, and increased lipogenic metabolism as a response to dieting and exercising.

Leptin, discovered in 1995, is a hormone which suppresses appetite. Produced primarily in fat tissue, leptin circulates generally in proportion to fat stores. It encourages people to stop eating when their fat cells are full. A newly discovered hormone (1999) called ghrelin seems to have the opposite effect. The hormone is a gastric hormone that has been identified as an endogenous ligand for the growth hormone (GH) secretagogue receptor subtype 1a (GHS-R1a), which stimulates growth hormone secretion in rats and humans (Kojima M *et al.*, *Nature*, 1999, 402: 656-60; Kojima M *et al.*, *Trends in Endocrinology and Metabolism*, 2001, 12: 118-22; Takaya K *et al.*, *J Clin Endocrinol Metab*, 2000, 85: 4908-11). Based on observation of orexigenic and adipogenic effects in rodents (Tschöp M *et al.*, *Nature*, 2000, 407: 908-913; Wren AM *et al.*, *Endocrinology*, 2000, 141: 4325-28; Nakazato M *et al.*, *Nature*, 2001, 409: 194-8; Shintani M *et al.*, *Diabetes*, 2001, 50: 227-232), an additional role for ghrelin in the regulation of energy balance has been assumed (Inui A, *Nature Reviews Neuroscience*, 2001, 2: 551-60; Horvath TL *et al.*, *Endocrinology*, 2001, 142(10): 4163-9). Studies have revealed that the infusion of ghrelin stimulates feeding and produces obesity in rodents (Tschöp M *et al.*, *Nature*, 2000, 407: 908-13) independently of changes in growth hormone secretion (Nakazato M *et al.*, *Nature*, 2001, vol. 409: 194-8). In humans subjects, infusion of ghrelin led to short-term increases in hunger (Wren AM *et al.*, *J Clin Endocrinol Metab*, 2001, 86: 5992). Cummings DE *et al.* (*N Engl J Med*, 2002, 346: 1623-30) reports that ghrelin levels rise just before meals and with food restriction or starvation and that they fall rapidly after meals. The authors hypothesize that the observed pre-meal increase triggers the desire to eat, and the increase in levels with long-term food restriction may contribute to hunger and possibly other adaptations that accompany negative energy balance. The theory is consistent with the evidence that ghrelin acts on hypothalamic neurons that are known to regulate energy balance (Nakazato M *et al.*, *Nature*, 2001, vol. 409: 194-8). Cummings DE *et al.* (2002) reported that the pre-meal plasma ghrelin levels increased upon diet-induced weight loss. The more weight an individual had lost, the bigger the post-diet increase in ghrelin levels. This is consistent with the hypothesis that ghrelin has a role in the long-term regulation of body weight. Furthermore, it was reported that gastric bypass surgery was associated with markedly suppressed ghrelin levels, possibly contributing to the weight-reducing effect of the by-pass patients. The by-pass surgery prevents the stomach cells from being exposed to food and this leads to a decrease in ghrelin production to levels almost not detectable (>75% reduction). Interestingly, most of the bypass patients reported a complete loss of interest in food subsequent to the operation, which may be due to the significant decrease in ghrelin production. Thus, ghrelin does play an important role in obesity and the need for a marked reduction in ghrelin production in obese patients undergoing dieting is essential for 1. losing weight *i.e.* reducing excess body fat, and 2. subsequently retaining a diet-induced weight loss.

Structure of ghrelin

Ghrelin shows a unique structure with an n-octanoyl ester in serine-3 residue. It has been shown that the first few residues of processed, mature ghrelin, Gly-Ser-Ser(n-octanoyl)-Phe segment constitute the active component of this peptide (Bednarek MA *et al.*, 2000).

5 Ghrelin's homology to other proteins

Ghrelin has been identified in the human stomach and is homologous to rat ghrelin apart from 2 amino acids. Human pre-proghrelin, isolated from a stomach cDNA library, consists of 117 amino acids. Rat and human pre-proghrelins are 82.9% identical. Ghrelin does not share high homology with any other non-ghrelin peptides identified so far.

10 Biological activity of ghrelin

Ghrelin is thought to alter feeding in part through the modulation of CNS control of gastric function (Date Y *et al.*, 2001; Masuda Y *et al.*, 2000). Specifically, ICV administration of ghrelin stimulated gastric acid secretion in a dose-dependent and atropine-sensitive manner. Immunohistochemistry demonstrated the induction of Fos expression in the nucleus of the solitary tract and dorsomotor nucleus of the rat vagus nerve. Asakawa A *et al.* showed in 2001 that ghrelin exhibited gastroprokinetic activity with structural resemblance to motilin and potent orexigenic (feeding) activity through action on the hypothalamic neuropeptide Y and Y(1) receptor, which was lost after vagotomy. Ghrelin decreased gastric vagal afferent discharge in contrast to other anorexigenic peptides that increased the activity. These authors and others (Toshinai K *et al.*, 2001) reported that ghrelin gene expression in the stomach was increased by fasting, insulin and in ob/ob mice.

In vivo demonstration of the role of ghrelin

As well as increasing GH release, exogenous ghrelin also increased food intake, caused weight gain and reduced fat utilization in mice and rats (Wren AM *et al.*, 2000; Tschöp M *et al.*, 2000). Likewise intracerebroventricular (ICV) administration of ghrelin also generated a dose-dependent increase in food intake and body weight. Rat serum ghrelin concentrations were increased by fasting and were reduced by re-feeding or oral glucose administration, but not by water ingestion. These authors proposed that ghrelin, in addition to its role in regulating GH secretion, alerts the hypothalamus when an increase in metabolic efficiency is necessary. Nakazato M *et al.* (2001) demonstrated that ghrelin is involved in the hypothalamic regulation of energy homeostasis. Intracerebroventricular injections of ghrelin strongly stimulated feeding in rats and increased body weight gain. Ghrelin also increased feeding in rats that were genetically deficient in GH. Anti-ghrelin immunoglobulin G robustly suppressed feeding. After

icv ghrelin administration, FOS protein, a marker of neuronal activation, was found in regions of primary importance in the regulation of feeding, including neuropeptide Y neurons and agouti-related protein neurons. Antibodies and antagonists of neuropeptide Y and agouti-related protein abolished ghrelin-induced feeding. Ghrelin augmented neuropeptide Y gene expression and blocked leptin-induced feeding reduction, implying that there is a competitive interaction between ghrelin and leptin in feeding regulation. It was therefore concluded that ghrelin is a physiologic mediator of feeding. In addition to animal studies ghrelin has also been investigated in a number of clinical studies. Levels of ghrelin demonstrated a two-fold increase 1 hour prior to eating and dropped to low levels within 1 hour of eating (see inset) suggesting that ghrelin plays an important role in the initiation of eating (Cummings DE *et al.*, 2001). This was confirmed by studies showing that ghrelin increased appetite and food intake in humans (Wren AM *et al.*, 2001).

Treatment of obesity at present and in the future

It is estimated that somewhere between 34 and 61 million people in the US are obese and in much of the developing world this incidence is increasing by about 1% per year. Following the withdrawal of early treatments, the market for anti-obesity pharmaceuticals was re-established in November 1997, when the FDA approved Abbott's sibutramine (Reductil/Meridia), for use in obesity, and still further in April 1999, when Roche's Xenical (orlistat) was also approved. The world obesity market has been predicted to reach \$3.7 billion by 2008 with a compound annual growth rate of 21.1%. This market potential has caused pharmaceutical companies to prioritise the identification of novel anti-obesity products and consequently the number of drugs in development has risen 3-fold over the past 7 years largely due to an increase in pre-clinical research activities. Pharmaceutical classes receiving greatest attention include 5-HT modulating drugs; beta 3 adrenoreceptor agonists; lipase inhibitors; melanocortin 4 agonists; and leptin agonists. Leptin agonists have created a storm of interest since this mediator is able to reduce feeding however recent observations that obese individuals produce high levels of and are resistant to leptin has driven the search for alternatives.

Ghrelin represents one of the most promising breaking targets in the field of obesity. Although scientists only identified ghrelin in 1999, more than 200 papers on the substance have already been published. Ghrelin acts to stimulate food intake but plasma levels are reduced in obese patients suggesting that this mediator represents a key regulator of food intake. Field-leaders currently believe that further reduction of ghrelin activity may offer a therapeutic target and hence antagonists of ghrelin receptor binding are emerging as a pharmacological option in the treatment of obesity. Correspondingly a number of tools are now available for the screening of ghrelin receptor antagonists. Despite the potential for drug discovery, ghrelin receptor antagonists have yet to appear although the publication of a number of patents suggests that such molecules may be on the way. Considering the proof of concept supporting

the development of ghrelin antagonists, the potential size of the obesity market and the relative paucity of treatments available to the clinician, now is an ideal time to invest in the development of this exciting therapeutic class.

Conditions where an increase in body fat are of interest.

- 5 Patients suffering from a number of diseases would benefit from an increase in body fat, quite contrary to the above-discussed obesity patients. Conditions do exist where the problem seems to be a lack of appetite and not insufficient access to food supplies. Such conditions include cachexia and anorexia.

OBJECT OF THE INVENTION

- 10 The object of the present invention is to provide novel therapies against conditions characterized by deposition of excess body fat resulting from energy intake exceeding energy expenditure as is characteristic for obesity. Another object is to provide therapies and treatments that induce an increase in body fat. A further object is to develop an autovaccine against ghrelin.

15 SUMMARY OF THE INVENTION

- Described herein is the use of an autovaccination technology for generating strong immune responses against an otherwise non-immunogenic self-protein, ghrelin, involved in excess body fat deposition. Thereby, a strong immune response is generated against ghrelin. Described is also the preparation of such vaccines for the prevention, possible cure or alleviation of the symptoms of such diseases associated with excess body fat deposits, but also for inducing an increase in body fat.
- 20

- The latter indication is the result of the surprising finding that a number of immunogenic ghrelin analogues of the present invention seem to effect an elevation in serum ghrelin in animals immunized with the variants. This elevation in serum ghrelin is accompanied by a significant increase in body weight in the immunized animals, even though the animal do not exhibit an increased intake in food.
- 25

- Further, the increase in circulating ghrelin levels render active immunization against autologous ghrelin an unexpected alternative to the direct administration of ghrelin in subjects that 1) would benefit from the physiological effects exerted by ghrelin and 2) do have the capacity to produce ghrelin themselves.
- 30

Thus, in its broadest and most general scope, the present invention relates to a method for inducing an immune response against autologous ghrelin in an animal, including a human being, the method comprising effecting presentation to the animal's immune system of an immunogenically effective amount of an immunogen selected from the group consisting of

- 5 - at least one ghrelin polypeptide or subsequence thereof which has been formulated so that immunization of the animal with the ghrelin polypeptide or subsequence thereof induces production of antibodies against the animal's autologous ghrelin, and
- at least one ghrelin analogue that incorporates into the same molecule at least one B-cell epitope of ghrelin and at least one chemical moiety not derived from ghrelin so
- 10 that immunization of the animal with the analogue induces production of antibodies against ghrelin.

As will appear from the discussion of the invention set forth below, the method can be used to either effect *in vivo* down-regulation of ghrelin activity or to effect *in vivo* up-regulation of ghrelin activity.

- 15 The most attractive aspect of this approach is that e.g. obesity can be controlled and/or reversed by periodic but not very frequent immunizations, in contrast to a therapeutic approach which involves administration of anti-ghrelin or molecules having a binding affinity to ghrelin analogous therewith. It is expected that 1-4 annual injections with an immunogenic composition according to the invention will be sufficient to obtain the desired effect, whereas admini-
- 20 stration of other inhibitors of ghrelin activity does or will require daily, or at least weekly, administrations. A similar advantage exists for indications where an increase in body fat is desired.

The invention also relates to ghrelin analogues as well as to nucleic acid fragments encoding a subset of these. Also immunogenic compositions comprising the analogues or the nucleic acid

25 fragments are part of the invention.

The invention also relates to a method of identifying analogues of ghrelin as well as a method for preparing a composition comprising the ghrelin analogues.

Finally, the invention also provides for passive immune therapy, where monoclonal anti-ghrelin antibodies are administered in order to obtain an effect similar to that of active vaccination.

30 tion.

LEGEND TO THE FIGURES

Fig. 1. Anti Ghrelin antibody titres in rats immunized against autologous Ghrelin.

Groups of rats (n=10) were vaccinated with different Ghrelin AutoVac peptides (peptide 3, 4, 5, corresponding to SEQ ID NOs: 15, 16, and 17, respectively), wildtype rat Ghrelin (peptide 2) or a negative control peptide derived from IgE (peptide 1). Pools of sera (after 3rd vaccination) from each of the five groups were tested for anti-Ghrelin antibody response in a direct ELISA, where the plate was coated with Ghrelin (Bachem, 2ug/ml). Sera were titrated three-fold with a start dilution of 1:10. As a negative control for non-specific binding of sera to the ELISA plate, sera from rats vaccinated with peptide 5 was added to non-coated wells (un-coated). The binding of anti-Ghrelin antibodies was detected with a HRP-conjugated anti-rat Ig secondary antibody (1:1000 dilution, Dako).

Fig. 2. Body weight of immunized rats.

The body weight was measured once a week in all groups from the initiation of the study at Time = 0.0. All groups are gaining weight in the 10 weeks of the study, but the weight gain is higher in animals with elevated levels of Ghrelin (Peptides 3-5) as compared to wt and control (Peptides 2 and 1, respectively)

Fig. 3. Food intake in immunized rats.

The food intake in the vaccinated rats was measured once a week in all groups from the initiation of the study at Time = 0.0. The graph shows the cumulative food intake over the 10 weeks duration of the study. Apparently, the demonstrated differences in body weight are not directly reflected in the cumulative food intake.

Fig. 4. Plasma ghrelin levels in vaccinated animals.

Blood samples drawn following 18h of fasting in order to normalise the Ghrelin levels. Pooled sera were analysed for Rat-Ghrelin using a commercial RIA kit from Phoenix (Ghrelin (Rat Mouse) - RIA Kit) according to the manufacturer's instructions. The first data from 11/4 are from a pre-bleed, while the remaining points are from bleeds taken 1 week after injections.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the following, a number of terms used in the present specification and claims will be defined and explained in detail in order to clarify the metes and bounds of the invention.

The term "immunogen" in the present context refers to an agent (a substance or a composition of matter) that induces an immune response. It will be understood that certain molecules

(e.g. traditional small haptens or self-protein that are tolerated in the autologous host) are incapable of inducing an immune response. However, some self-proteins are, when formulated in very strong immunologic adjuvants, capable of inducing an immune response in spite of the normally tolerant state of the immunized animal. In such a context, the "immunogen" is therefore the composition of matter (self-protein with adjuvant) and not just a single molecule.

The terms "T-lymphocyte" and "T-cell" will be used interchangeably for lymphocytes of thymic origin which are responsible for various cell mediated immune responses as well as for helper activity in the humoral immune response. Likewise, the terms "B-lymphocyte" and "B-cell" will be used interchangeably for antibody-producing lymphocytes.

A "ghrelin polypeptide" is herein intended to denote polypeptides having the amino acid sequence of the above-discussed ghrelin proteins derived from humans and other mammals (or truncates thereof sharing a substantial amount of B-cell epitopes with intact ghrelin), but also polypeptides having the amino acid sequence identical to xeno-analogues of these proteins isolated from other species are embraced by the term; included in the term is both the mature ghrelin peptide as well as the ghrelin propeptide and the ghrelin pre-propeptide. Also unglycosylated forms of ghrelin which are prepared in prokaryotic system are included within the boundaries of the term as are forms having varying glycosylation patterns due to the use of e.g. yeasts or other non-mammalian eukaryotic expression systems. It should, however, be noted that when using the term "a ghrelin polypeptide" it is intended that the polypeptide in question is normally non-immunogenic when presented to the animal to be treated. In other words, the ghrelin polypeptide is a self-protein or is a xeno-analogue of such a self-protein which will not normally give rise to an immune response against ghrelin of the animal in question.

A "ghrelin analogue" is a ghrelin polypeptide which has been subjected to changes in its primary structure. Such a change can e.g. be in the form of fusion of a ghrelin polypeptide to a suitable fusion partner (i.e. a change in primary structure exclusively involving C- and/or N-terminal additions of amino acid residues) and/or it can be in the form of insertions and/or deletions and/or substitutions in the ghrelin polypeptide's amino acid sequence. Also encompassed by the term are derivatized ghrelin molecules, cf. the discussion below of modifications of ghrelin.

It should be noted that the use as a vaccine in a human of e.g. a canine analogue of human ghrelin could be imagined to produce the desired immunity against ghrelin. Such use of a xeno-analogue for immunization is also considered to be a "ghrelin analogue" as defined above.

When using the abbreviation "ghrelin" herein, this is intended as a reference to the amino acid sequence of wildtype ghrelin (also denoted "ghrelin" and "ghrelin-wt" herein). This term embraces both the propeptide and the mature peptide, so mature ghrelin is termed ghrelin-m. Mature human ghrelin is denoted h-ghrelin, h-ghrelin-m, and murine mature ghrelin is denoted m-ghrelin, m-ghrelin-m, or m-ghrelin-wt, etc. In cases where a DNA construct includes information encoding a leader sequence or other material, this will normally be clear from the context.

The term "polypeptide" is in the present context intended to mean both short peptides of from 2 to 10 amino acid residues, oligopeptides of from 11 to 100 amino acid residues, and polypeptides of more than 100 amino acid residues. Furthermore, the term is also intended to include proteins, *i.e.* functional biomolecules comprising at least one polypeptide; when comprising at least two polypeptides, these may form complexes, be covalently linked, or may be non-covalently linked. The polypeptide(s) in a protein can be glycosylated and/or lipidated and/or comprise prosthetic groups. Also, the term "polyamino acid" is an equivalent to the term "polypeptide".

The term "subsequence" means any consecutive stretch of at least 3 amino acids or, when relevant, of at least 3 nucleotides, derived directly from a naturally occurring ghrelin amino acid sequence or nucleic acid sequence, respectively.

The term "animal" is in the present context in general intended to denote an animal species (preferably mammalian), such as *Homo sapiens*, *Canis domesticus*, etc. and not just one single animal. However, the term also denotes a population of such an animal species, since it is important that the individuals immunized according to the method of the invention all harbour substantially the same ghrelin allowing for immunization of the animals with the same immunogen(s). If, for instance, genetic variants of ghrelin exist in different human population it may be necessary to use different immunogens in these different populations in order to be able to break the autotolerance towards ghrelin in each population. It will be clear to the skilled person that an animal in the present context is a living being which has an immune system. It is preferred that the animal is a vertebrate, such as a mammal.

By the term "*in vivo* down-regulation of ghrelin activity" is herein meant reduction in the living organism of the number of interactions between ghrelin and its receptors (or between ghrelin and other possible biologically important binding partners for this molecule). The down-regulation can be obtained by means of several mechanisms: Of these, simple interference with the active site in ghrelin by antibody binding is the most simple. However, it is also within the scope of the present invention that the antibody binding results in removal of ghrelin by scavenger cells (such as macrophages and other phagocytic cells). Another possibility is

binding of anti-ghrelin antibodies that are capable of interfering with the normal cleavage of proghrelin that result in mature ghrelin.

The expression "effecting presentation ... to the immune system" is intended to denote that the animal's immune system is subjected to an immunogenic challenge in a controlled manner. As will appear from the disclosure below, such challenge of the immune system can be effected in a number of ways of which the most important are vaccination with polypeptide containing "pharmaccines" (*i.e.* a vaccine which is administered to treat or ameliorate ongoing disease) or nucleic acid "pharmaccine" vaccination. The important result to achieve is that immune competent cells in the animal are confronted with the antigen in an immunologically effective manner, whereas the precise mode of achieving this result is of less importance to the inventive idea underlying the present invention.

The term "immunogenically effective amount" has its usual meaning in the art of immunology, *i.e.* an amount of an immunogen which is capable of inducing an immune response which significantly engages molecules which share immunological features with the immunogen.

When using the expression that the ghrelin has been "modified" is herein meant a chemical modification of the polypeptide which constitutes the backbone of ghrelin. Such a modification can *e.g.* be derivatization (*e.g.* alkylation, acylation, esterification etc.) of certain amino acid residues in the ghrelin sequence, but as will be appreciated from the disclosure below, the preferred modifications comprise changes of (or additions to) the primary structure of the ghrelin amino acid sequence. One particular modification is the omission of the naturally occurring *n*-octanoyl group in ghrelin.

When discussing "autotolerance towards ghrelin" it is understood that since ghrelin is a self-protein in the population to be vaccinated, normal individuals in the population do not mount an immune response against ghrelin; it cannot be excluded, though, that occasional individuals in an animal population might be able to produce antibodies against native ghrelin, *e.g.* as part of an autoimmune disorder. At any rate, an animal will normally only be autotolerant towards its own ghrelin, but it cannot be excluded that ghrelin analogues derived from other animal species or from a population having a different ghrelin phenotype would also be tolerated by said animal.

A "foreign T-cell epitope" (or: "foreign T-lymphocyte epitope") is a peptide which is able to bind to an MHC molecule and which stimulates T-cells in an animal species. Preferred foreign T-cell epitopes in the invention are "promiscuous" epitopes, *i.e.* epitopes which bind to a substantial fraction of a particular class of MHC molecules in an animal species or population. Only a very limited number of such promiscuous T-cell epitopes are known, and they will be discussed in detail below. It should be noted that in order for the immunogens which are used

according to the present invention to be effective in as large a fraction of an animal population as possible, it may be necessary to 1) insert several foreign T-cell epitopes in the same ghrelin analogue or 2) prepare several ghrelin analogues wherein each analogue has a different promiscuous epitope inserted. It should be noted also that the concept of foreign T-cell epitopes also encompasses use of cryptic T-cell epitopes, *i.e.* epitopes which are derived from a self-protein and which only exerts immunogenic behaviour when existing in isolated form without being part of the self-protein in question.

A "foreign T helper lymphocyte epitope" (a foreign T_H epitope) is a foreign T cell epitope which binds an MHC Class II molecule and can be presented on the surface of an antigen presenting cell (APC) bound to the MHC Class II molecule.

A "functional part" of a (bio)molecule is in the present context intended to mean the part of the molecule which is responsible for at least one of the biochemical or physiological effects exerted by the molecule. It is well-known in the art that many enzymes and other effector molecules have an active site which is responsible for the effects exerted by the molecule in question. Other parts of the molecule may serve a stabilizing or solubility enhancing purpose and can therefore be left out if these purposes are not of relevance in the context of a certain embodiment of the present invention. For instance it is possible to use certain cytokines as a modifying moiety in ghrelin (*cf.* the detailed discussion below), and in such a case, the issue of stability may be irrelevant since the coupling to ghrelin provides the stability necessary.

The term "adjuvant" has its usual meaning in the art of vaccine technology, *i.e.* a substance or a composition of matter which is 1) not in itself capable of mounting a specific immune response against the immunogen of the vaccine, but which is 2) nevertheless capable of enhancing the immune response against the immunogen. Or, in other words, vaccination with the adjuvant alone does not provide an immune response against the immunogen, vaccination with the immunogen may or may not give rise to an immune response against the immunogen, but the combination of vaccination with immunogen and adjuvant induces an immune response against the immunogen which is stronger than that induced by the immunogen alone.

"Targeting" of a molecule is in the present context intended to denote the situation where a molecule upon introduction in the animal will appear preferentially in certain tissue(s) or will be preferentially associated with certain cells or cell types. The effect can be accomplished in a number of ways including formulation of the molecule in composition facilitating targeting or by introduction in the molecule of groups which facilitates targeting. These issues will be discussed in detail below.

"Stimulation of the Immune system" means that a substance or composition of matter exhibits a general, non-specific immunostimulatory effect. A number of adjuvants and putative adjuvants (such as certain cytokines) share the ability to stimulate the immune system. The result of using an immunostimulating agent is an increased "alertness" of the immune system meaning that simultaneous or subsequent immunization with an immunogen induces a significantly more effective immune response compared to isolated use of the immunogen.

"Productive binding" means binding of a peptide to the MHC molecule (Class I or II) so as to be able to stimulate T-cells that engage a cell that present the peptide bound to the MHC molecule. For instance, a peptide bound to an MHC Class II molecule on the surface of an APC is said to be productively bound if this APC will stimulate a T_H cell that binds to the presented peptide-MHC Class II complex.

Preferred embodiments of anti-ghrelin immunization

As mentioned briefly above, the immunization against ghrelin can be active as well as passive. Even though the focus of the present invention is the administration of agents that induce an active immune response against ghrelin, it is also within the scope of the present invention to administer agents that bind ghrelin in vivo. For instance, it is possible to utilise the well-known technology of monoclonal antibody administration in the present invention. It is in this context preferred to use humanized or completely human monoclonal antibodies, e.g. by employing transgenic mice that express human immunoglobulin light and heavy chains. The skilled person will know how to dose and administer such antibody compositions. Alternatively, a soluble version of a ghrelin receptor can be injected, resulting in an effective binding in the bloodstream to ghrelin.

However, as mentioned, the preferred embodiment entails active immunization against ghrelin.

It is preferred that the ghrelin polypeptide used as an immunogen in the method of the invention is a modified molecule wherein at least one change is present in the ghrelin polypeptide amino acid sequence, since the chances of obtaining the all-important breaking of autotolerance towards ghrelin is greatly facilitated that way. It should be noted that this does not exclude the possibility of using such a modified ghrelin in formulations which further facilitate the breaking of autotolerance against ghrelin, e.g. formulations containing certain adjuvants discussed in detail below.

It has been shown (in Dalum I et al., 1996, J. Immunol. **157**: 4796-4804) that potentially self-reactive B-lymphocytes recognizing self-proteins are physiologically present in normal individuals. However, in order for these B-lymphocytes to be induced to actually produce anti-

bodies reactive with the relevant self-proteins, assistance is needed from cytokine producing T-helper lymphocytes (T_H -cells or T_H -lymphocytes). Normally this help is not provided because T-lymphocytes in general do not recognize T-cell epitopes derived from self-proteins when presented by antigen presenting cells (APCs). However, by providing an element of "foreign-
5 ness" in a self-protein (*i.e.* by introducing an immunologically significant modification), T-cells recognizing the foreign element are activated upon recognizing the foreign epitope on an APC (such as, initially, a mononuclear cell). Polyclonal B-lymphocytes (which are also specialised APCs) capable of recognising self-epitopes on the modified self-protein also internalise the antigen and subsequently presents the foreign T-cell epitope(s) thereof, and the activated T-
10 lymphocytes subsequently provide cytokine help to these self-reactive polyclonal B-lymphocytes. Since the antibodies produced by these polyclonal B-lymphocytes are reactive with different epitopes on the modified polypeptide, including those which are also present in the native polypeptide, an antibody cross-reactive with the non-modified self-protein is induced. In conclusion, the T-lymphocytes can be led to act as if the population of polyclonal
15 B-lymphocytes have recognised an entirely foreign antigen, whereas in fact only the inserted epitope(s) is/are foreign to the host. In this way, antibodies capable of *cross-reacting* with non-modified self-antigens are induced.

Several ways of modifying a peptide self-antigen in order to obtain breaking of autotolerance are known in the art. Hence, according to the invention, the modification can include that

- 20 - at least one foreign T-cell epitope is introduced, and/or
- at least one first moiety is introduced which effects targeting of the modified molecule to an antigen presenting cell (APC), and/or
- at least one second moiety is introduced which stimulates the immune system, and/or
- at least one third moiety is introduced which optimises presentation of the modified
25 ghrelin polypeptide to the immune system.

However, all these modifications should be carried out while maintaining a substantial fraction of the original B-lymphocyte epitopes in ghrelin, since the B-lymphocyte recognition of the native molecule is thereby enhanced.

In one preferred embodiment, side groups (in the form of foreign T-cell epitopes or the
30 above-mentioned first, second and third moieties) are covalently or non-covalently introduced. This is intended to mean that stretches of amino acid residues derived from ghrelin are derivatized without altering the primary amino acid sequence, or at least without introducing changes in the peptide bonds between the individual amino acids in the chain.

An alternative, and preferred, embodiment utilises amino acid substitution and/or deletion and/or insertion and/or addition (which may be effected by recombinant means or by means of peptide synthesis; modifications which involve longer stretches of amino acids can give rise to fusion polypeptides). One especially preferred version of this embodiment is the technique described in WO 95/05849, which discloses a method for immunizing against self-proteins by immunising with analogues of the self-proteins wherein a number of amino acid sequence(s) has been substituted with a corresponding number of amino acid sequence(s), which each comprise a foreign immunodominant T-cell epitope, while at the same time maintaining the overall 3 dimensional structure of the self-protein in the analogue. For the purposes of the present invention, it is however sufficient if the modification (be it an amino acid insertion, addition, deletion or substitution) gives rise to a foreign T-cell epitope and at the same time preserves a substantial number of the B-cell epitopes in ghrelin. However, in order to obtain maximum efficacy of the immune response induced, it is preferred that the overall tertiary structure of ghrelin is maintained in the modified molecule.

15 The following formula describes the ghrelin constructs generally covered by the invention:

The following formula describes the molecular constructs generally covered by the invention:

$$(\text{MOD}_1)_{s1}(\text{ghr}_{e1})_{n1}(\text{MOD}_2)_{s2}(\text{ghr}_{e2})_{n2} \dots (\text{MOD}_x)_{sx}(\text{ghr}_{ex})_{nx} \quad (\text{I})$$

-where ghr_{e1} - ghr_{ex} are x B-cell epitope containing subsequences of a ghrelin polypeptide, which independently are identical or non-identical and which may contain or not contain foreign side groups, x is an integer ≥ 3 , $n1$ - n_x are x integers ≥ 0 (at least one is ≥ 1), MOD_1 - MOD_x are x modifications introduced between the preserved B-cell epitopes, and $s1$ - s_x are x integers ≥ 0 (at least one is ≥ 1 if no side groups are introduced in the ghr_{ex} sequences). Thus, given the general functional restraints on the immunogenicity of the constructs, the invention allows for all kinds of permutations of the original sequence of the ghrelin polypeptide, and all kinds of modifications therein. Thus, included in the invention are modified ghrelin polypeptides obtained by omission of parts of the sequence of the ghrelin polypeptide, which e.g. exhibit adverse effects *in vivo* and thus could give rise to undesired immunological reactions.

One preferred embodiment of the invention utilises multiple presentations of B-lymphocyte epitopes of the ghrelin polypeptide (i.e. formula I wherein at least one B-cell epitope is present in two positions). This effect can be achieved in various ways, e.g. by simply preparing fusion polypeptides comprising the structure (ghrelin polypeptide)_m, where m is an integer ≥ 2 and then introduce the modifications discussed herein in at least one of the ghrelin sequences. It is preferred that the modifications introduced includes at least one duplication of a B-lymphocyte epitope and/or the introduction of a hapten. These embodiments including multi-

ple presentations of selected epitopes are especially preferred in situations where merely minor parts of the ghrelin polypeptide are useful as constituents in a vaccine agent.

As mentioned above, the introduction of a foreign T-cell epitope can be accomplished by introduction of at least one amino acid insertion, addition, deletion, or substitution. Of course, the normal situation will be the introduction of more than one change in the amino acid sequence (e.g. Insertion of or substitution by a complete T-cell epitope) but the important goal to reach is that the analogue, when processed by an antigen presenting cell (APC), will give rise to such a foreign immunodominant T-cell epitope being presented in context of an MHC Class II molecule on the surface of the APC. Thus, if the amino acid sequence of the ghrelin polypeptide in appropriate positions comprises a number of amino acid residues which can also be found in a foreign T_H epitope then the introduction of a foreign T_H epitope can be accomplished by providing the remaining amino acids of the foreign epitope by means of amino acid insertion, addition, deletion and substitution. In other words, it is not necessary to introduce a complete T_H epitope by insertion or substitution in order to fulfil the purpose of the present invention.

It is preferred that the number of amino acid insertions, deletions, substitutions or additions is at least 2, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 and 25 insertions, substitutions, additions or deletions. It is furthermore preferred that the number of amino acid insertions, substitutions, additions or deletions is not in excess of 150, such as at most 100, at most 90, at most 80, and at most 70. It is especially preferred that the number of substitutions, insertions, deletions, or additions does not exceed 60, and in particular the number should not exceed 50 or even 40. Most preferred is a number of not more than 30. With respect to amino acid additions, it should be noted that these, when the resulting construct is in the form of a fusion polypeptide, is often considerably higher than 150.

Preferred embodiments of the invention include modification by introducing at least one foreign immunodominant T-cell epitope. It will be understood that the question of immune dominance of a T-cell epitope depends on the animal species in question. As used herein, the term "immunodominance" simply refers to epitopes which in the vaccinated individual/population gives rise to a significant immune response, but it is a well-known fact that a T-cell epitope which is immunodominant in one individual/population is not necessarily immunodominant in another individual of the same species, even though it may be capable of binding MHC-II molecules in the latter individual. Hence, for the purposes of the present invention, an immune dominant T-cell epitope is a T-cell epitope which will be effective in providing T-cell help when present in an antigen. Typically, immune dominant T-cell epitopes have as an inherent feature that they will substantially always be presented bound to an MHC Class II molecule, irrespective of the polypeptide wherein they appear.

Another important point is the issue of MHC restriction of T-cell epitopes. In general, naturally occurring T-cell epitopes are MHC restricted, *i.e.* a certain peptides constituting a T-cell epitope will only bind effectively to a subset of MHC Class II molecules. This in turn has the effect that in most cases the use of one specific T-cell epitope will result in a vaccine component which is only effective in a fraction of the population, and depending on the size of that fraction, it can be necessary to include more T-cell epitopes in the same molecule, or alternatively prepare a multi-component vaccine wherein the components are variants of the ghrelin polypeptide which are distinguished from each other by the nature of the T-cell epitope introduced.

- 10 If the MHC restriction of the T-cells used is completely unknown (for instance in a situation where the vaccinated animal has a poorly defined MHC composition), the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula

$$(II) \quad f_{\text{population}} = 1 - \prod_{i=1}^n (1 - p_i)$$

- 15 -where p_i is the frequency in the population of responders to the i^{th} foreign T-cell epitope present in the vaccine composition, and n is the total number of foreign T-cell epitopes in the vaccine composition. Thus, a vaccine composition containing 3 foreign T-cell epitopes having response frequencies in the population of 0.8, 0.7, and 0.6, respectively, would give

$$1 - 0.2 \times 0.3 \times 0.4 = 0.976$$

- 20 -*i.e.* 97.6 percent of the population will statistically mount an MHC-II mediated response to the vaccine.

- The above formula does not apply in situations where a more or less precise MHC restriction pattern of the peptides used is known. If, for instance a certain peptide only binds the human MHC-II molecules encoded by HLA-DR alleles DR1, DR3, DR5, and DR7, then the use of this peptide together with another peptide which binds the remaining MHC-II molecules encoded by HLA-DR alleles will accomplish 100% coverage in the population in question. Likewise, if the second peptide only binds DR3 and DR5, the addition of this peptide will not increase the coverage at all. If one bases the calculation of population response purely on MHC restriction of T-cell epitopes in the vaccine, the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula:

$$(III) \quad f_{\text{population}} = 1 - \prod_{j=1}^3 (1 - \varphi_j)^2$$

-wherein φ_j is the sum of frequencies in the population of allelic haplotypes encoding MHC molecules which bind any one of the T-cell epitopes in the vaccine and which belong to the j^{th} of the 3 known HLA loci (DP, DR and DQ); In practice, it is first determined which MHC molecules will recognize each T-cell epitope in the vaccine and thereafter these are listed by type
 5 (DP, DR and DQ) - then, the individual frequencies of the different listed allelic haplotypes are summed for each type, thereby yielding φ_1 , φ_2 , and φ_3 .

It may occur that the value p_i in formula II exceeds the corresponding theoretical value n_i :

$$(IV) \quad \pi_i = 1 - \prod_{j=1}^3 (1 - \nu_j)^2$$

-wherein ν_j is the sum of frequencies in the population of allelic haplotype encoding MHC molecules which bind the i^{th} T-cell epitope in the vaccine and which belong to the j^{th} of the 3 known HLA loci (DP, DR and DQ). This means that in $1 - \pi_i$ of the population is a frequency of responders of $f_{\text{residual}_i} = (p_i - n_i) / (1 - n_i)$. Therefore, formula III can be adjusted so as to yield formula V:

$$(V) \quad f_{\text{population}} = 1 - \prod_{j=1}^3 (1 - \varphi_j)^2 + \left(1 - \prod_{i=1}^n (1 - f_{\text{residual}_i}) \right)$$

15 -where the term $1 - f_{\text{residual}_i}$ is set to zero if negative. It should be noted that formula V requires that all epitopes have been haplotype mapped against identical sets of haplotypes.

Therefore, when selecting T-cell epitopes to be introduced in the analogue, it is important to include all knowledge of the epitopes which is available: 1) The frequency of responders in the population to each epitope, 2) MHC restriction data, and 3) frequency in the population of the
 20 relevant haplotypes.

There exist a number of naturally occurring "promiscuous" T-cell epitopes which are active in a large proportion of individuals of an animal species or an animal population and these are preferably introduced in the vaccine thereby reducing the need for a very large number of different analogues in the same vaccine.

25 The promiscuous epitope can according to the invention be a naturally occurring human T-cell epitope such as epitopes from tetanus toxoid (e.g. the P2 and P30 epitopes), diphtheria toxoid, Influenza virus hemagglutinin (HA), and *P. falciparum* CS antigen.

Over the years a number of other promiscuous T-cell epitopes have been identified. Especially peptides capable of binding a large proportion of HLA-DR molecules encoded by the different
 30 HLA-DR alleles have been identified and these are all possible T-cell epitopes to be introduced in the analogues used according to the present invention. Cf. also the epitopes discussed in

the following references which are hereby all incorporated by reference herein: WO 98/23635 (Frazer IH et al., assigned to The University of Queensland); Southwood S et al., 1998, J. Immunol. **160**: 3363-3373; Sinigaglia F et al., 1988, Nature **336**: 778-780; Chicz RM et al., 1993, J. Exp. Med **178**: 27-47; Hammer J et al., 1993, Cell **74**: 197-203; and Falk K et al.,
5 1994, Immunogenetics **39**: 230-242. The latter reference also deals with HLA-DQ and -DP ligands. All epitopes listed in these 5 references are relevant as candidate natural epitopes to be used in the present invention, as are epitopes which share common motifs with these.

Alternatively, the epitope can be any artificial T-cell epitope which is capable of binding a large proportion of MHC Class II molecules. In this context the pan DR epitope peptides
10 ("PADRE") described in WO 95/07707 and in the corresponding paper Alexander J et al., 1994, Immunity **1**: 751-761 (both disclosures are incorporated by reference herein) are interesting candidates for epitopes to be used according to the present invention. It should be noted that the most effective PADRE peptides disclosed in these papers carry D-amino acids in the C- and N-termini in order to improve stability when administered. However, the present
15 invention primarily aims at incorporating the relevant epitopes as part of the modified ghrelin polypeptide, which should then subsequently be broken down enzymatically inside the lysosomal compartment of APCs to allow subsequent presentation in the context of an MHC-II molecule and therefore it is not expedient to incorporate D-amino acids in the epitopes used in the present invention.

20 One especially preferred PADRE peptide is the one having the amino acid sequence AKFVAAWTLKAAA or an immunologically effective subsequence thereof. This, and other epitopes having the same lack of MHC restriction are preferred T-cell epitopes which should be present in the analogues used in the inventive method. Such super-promiscuous epitopes will allow for the most simple embodiments of the invention wherein only one single modified
25 ghrelin polypeptide is presented to the vaccinated animal's immune system.

As mentioned above, the modification of the ghrelin polypeptide can also include the introduction of a first moiety which targets the modified ghrelin polypeptide to an APC or a B-lymphocyte. For instance, the first moiety can be a specific binding partner for a B-lymphocyte specific surface antigen or for an APC specific surface antigen. Many such specific surface an-
30 tigens are known in the art. For instance, the moiety can be a carbohydrate for which there is a receptor on the B-lymphocyte or the APC (e.g. mannan or mannose). Alternatively, the second moiety can be a hapten. Also an antibody fragment which specifically recognizes a surface molecule on APCs or lymphocytes can be used as a first moiety (the surface molecule can e.g. be an FCγ receptor of macrophages and monocytes, such as FCγRI or, alternatively any
35 other specific surface marker such as CD40 or CTLA-4). It should be noted that all these exemplary targeting molecules can be used as part of an adjuvant also, cf. below.

As an alternative or supplement to targeting the modified ghrelin polypeptide to a certain cell type in order to achieve an enhanced immune response, it is possible to increase the level of responsiveness of the immune system by including the above-mentioned second moiety which stimulates the immune system. Typical examples of such second moieties are cytokines, and
5 heat-shock proteins or molecular chaperones, as well as effective parts thereof.

Suitable cytokines to be used according to the invention are those which will normally also function as adjuvants in a vaccine composition, *i.e.* for instance interferon γ (IFN- γ), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and granulocyte-macrophage colony
10 stimulating factor (GM-CSF); alternatively, the functional part of the cytokine molecule may suffice as the second moiety. With respect to the use of such cytokines as adjuvant substances, cf. the discussion below.

According to the invention, suitable heat-shock proteins or molecular chaperones used as the second moiety can be HSP70 (heat shock protein 70), HSP90 (heat shock protein 90), HSC70
15 (heat shock cognate protein 70), GRP94 (also known as gp96, cf. Wearsch PA *et al.* 1998, *Biochemistry* **37**: 5709-19), and CRT (calreticulin).

Alternatively, the second moiety can be a toxin, such as listeriolysin (LLO), lipid A and heat-labile enterotoxin. Also, a number of mycobacterial derivatives such as MDP (muramyl dipeptide), CFA (complete Freund's adjuvant) and the trehalose diesters TDM and TDE are inter-
20 esting possibilities.

Also the possibility of introducing a third moiety which enhances the presentation of the modified ghrelin polypeptide to the immune system is an important embodiment of the invention. The art has shown several examples of this principle. For instance, it is known that the palmitoyl lipidation anchor in the *Borrelia burgdorferi* protein OspA can be utilised so as to
25 provide self-adjuvating polypeptides (cf. *e.g.* WO 96/40718) - It seems that the lipidated proteins form up micelle-like structures with a core consisting of the lipidation anchor parts of the polypeptides and the remaining parts of the molecule protruding there from, resulting in multiple presentations of the antigenic determinants. Hence, the use of this and related approaches using different lipidation anchors (*e.g.* a myristyl group, a myristyl group, a farnesyl
30 group, a geranyl-geranyl group, a GPI-anchor, and an N-acyl diglyceride group) are preferred embodiments of the invention, especially since the provision of such a lipidation anchor in a recombinantly produced protein is fairly straightforward and merely requires use of *e.g.* a naturally occurring signal sequence as a fusion partner for the modified ghrelin polypeptide. Another possibility is use of the C3d fragment of complement factor C3 or C3 itself (cf.
35 Dempsey *et al.*, 1996, *Science* **271**, 348-350 and Lou & Kohler, 1998, *Nature Biotechnology* **16**, 458-462).

Another attractive way of presenting multiple copies of epitopic regions is the technology disclosed in WO 00/32227, where antigens are presented in ordered, repetitive patterns, thereby giving rise to T-cell Independent Immunogens that resemble virus capsids. In the context of the present invention, the technology of WO 00/32227 is regarded as application of a specialized adjuvant. The disclosure of WO 00/32227 is hereby incorporated by reference herein. An alternative embodiment of the invention which also results in the preferred presentation of multiple (e.g. at least 2) copies of the important epitopic regions of the ghrelin polypeptide to the immune system is the covalent coupling of polyamino acids selected from the ghrelin polypeptide, the subsequence thereof, or the analogues thereof to certain molecules and, when necessary, together with foreign T_H epitopes or one of the first, second or third moieties discussed above. For instance, polymers can be used, e.g. polyhydroxypolymers, notably carbohydrates such as dextran, cf. e.g. Lees A *et al.*, 1994, Vaccine **12**: 1160-1166; Lees A *et al.*, 1990, J Immunol. **145**: 3594-3600, but also mannose and mannan are useful alternatives. Integral membrane proteins from e.g. *E. coli* and other bacteria are also useful conjugation partners. The traditional carrier molecules such as keyhole limpet hemocyanin (KLH), tetanus toxoid, diphtheria toxoid, and bovine serum albumin (BSA) are also preferred and useful conjugation partners. Preferred embodiments of covalent coupling of the ghrelin polypeptide to pharmaceutically acceptable polyhydroxypolymers such as carbohydrates involve the use of at least one ghrelin polypeptide and at least one foreign T-helper epitope which are coupled *separately* to the polyhydroxypolymer (*i.e.* the foreign T-helper epitope and the ghrelin polypeptide are not fused to each other but rather bound to the polyhydroxypolymer which then serves as a carrier backbone). Again, such an embodiment is most preferred when the suitable B-cell epitope carrying regions of the ghrelin polypeptide are constituted by short peptide stretches – this is because this approach is one very convenient way to achieve multiple presentations of selected epitopes in the resulting immunogenic agent.

It is especially preferred that the coupling of the polyamino acids to the polyhydroxypolymer is by means of an amide bond which can be cleaved by a peptidase. This strategy has the effect that APCs will be able to take up the conjugate and at the same time be able to process the conjugate and subsequently present the foreign T-cell epitope in an MHC Class II context.

One way of achieving coupling of peptides (both the ghrelin polypeptide of interest as well as the foreign epitope) is to activate a suitable polyhydroxypolymer with tresyl (trifluoroethylsulphonyl) groups or other suitable activation groups such as maleimido, p-Nitrophenyl chloroformate (for activation of OH groups and formation of a peptide bond between peptide and polyhydroxypolymer), and tosyl (p-toluenesulfonyl). It is e.g. possible to prepare activated polysaccharides as described in WO 00/05316 and US 5,874,469 (both incorporated by reference herein) and couple these to ghrelin peptides and T-cell epitopes prepared by means of conventional solid or liquid phase peptide synthesis techniques. The resulting product consists of a polyhydroxypolymer backbone (e.g. a dextran backbone) that has, attached thereto by

their N-termini or by other available nitrogen moieties, ghrelin polypeptides and foreign T-cell epitopes. If desired, it is possible to synthesize the ghrelin polypeptides so as to protect all available amino groups but the one at the N-terminus, subsequently couple the resulting protected peptides to the trisylated dextran moiety, and finally de-protecting the resulting
5 conjugate. A specific example of this approach is described in the examples below.

Instead of using the water-soluble polysaccharide molecules as taught in WO 00/05316 and US 5,874,469, it is equally possible to utilise cross-linked polysaccharide molecules, thereby obtaining a particulate conjugate between polypeptides and polysaccharide – this is believed to lead to an improved presentation to the immune system of the polypeptides, since two
10 goals are reached, namely to obtain a local deposit effect when injecting the conjugate and to obtain particles which are attractive targets for APCs. The approach of using such particulate systems is also detailed in the examples.

Considerations underlying chosen areas of introducing modifications in ghrelin polypeptides are a) preservation of known and predicted B-cell epitopes, b) preservation of 3D structure, c)
15 avoidance of B-cell epitopes present on "producer cells" etc. At any rate, as discussed above, it is fairly easy to screen a set of modified ghrelin molecules which have all been subjected to introduction of a T-cell epitope in different locations.

Vaccination targeting both the mature form of ghrelin and the propeptide form can be envisaged and both forms are believed to entail distinct advantages. By targeting the propeptide it
20 should be possible to interfere with the enzymatic processing that leads to formation of mature ghrelin. If the immunogen used includes B-cell epitopes from both mature ghrelin and from proghrelin, then the antibodies formed would have the maximum capacity for down-regulation of mature ghrelin: Not only would it be possible to neutralize mature ghrelin, but also its formation would be reduced since the enzymatic processing would be inhibited be-
25 cause strongly binding antibodies would "mask" the cleavage site and other sites important for the enzymatic cleavage to take place.

If, on the other hand, it were desired to reduce the level of mature ghrelin to a lesser extent, then it would be preferable to vaccinate against the part of the propeptide that does not in-
clude substantial amounts of the mature ghrelin. By doing so, the mature ghrelin would not
30 be bound by antibodies, but only the formation of mature ghrelin would be reduced.

Finally, if it were only of interest to target the mature molecule (which has very little sequence identity with any known non-ghrelins) then the immunogen should predominantly include B-cell epitopes from the mature ghrelin.

Since the most preferred embodiments of the present invention involve immunization against of human ghrelin, it is consequently preferred that the ghrelin polypeptide discussed above is a human ghrelin polypeptide – however, any discussions below of human ghrelin could be used for ghrelin from other species, notably those listed in the sequence listing of this application. It will then be understood that teachings relating to changes in the human sequence should be transposed to the relevant sequence in the relevant animal: From the sequence listing it appears where the boundaries for the mature ghrelin peptide sequence can be found, and it will be understood that any specific sequence data referred to in the human sequence should take offset in the corresponding sequences in the various mammalian ghrelin sequences.

In the embodiments relating to human ghrelin, it is especially preferred that the human ghrelin polypeptide has been modified by substituting at least one amino acid sequence in SEQ ID NO: 11 with at least one amino acid sequence of equal or different length and containing a foreign T_H epitope. Alternatively, the foreign T_H epitope may simply be inserted in SEQ ID NO: 11.

More specifically, a T_H containing (or completing) amino acid sequence which is introduced into SEQ ID NO: 11 may be introduced at any amino acid in SEQ ID NO: 11. That is, the introduction is possible after any one of amino acids 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, and 117 in SEQ ID NO: 11, and, in case of an addition, also before amino acid 1. This may be accompanied by deletion of amino acid(s) 1 and/or 2 and/or 3 and/or 4 and/or 5 and/or 6 and/or 7 and/or 8 and/or 9 and/or 10 and/or 11 and/or 12 and/or 13 and/or 14 and/or 15 and/or 16 and/or 17 and/or 18 and/or 19 and/or 20 and/or 21 and/or 22 and/or 23 and/or 24 and/or 25 and/or 26 and/or 27 and/or 28 and/or 29 and/or 30 and/or 31 and/or 32 and/or 33 and/or 34 and/or 35 and/or 36 and/or 37 and/or 38 and/or 39 and/or 40 and/or 41 and/or 42 and/or 43 and/or 44 and/or 45 and/or 46 and/or 47 and/or 48 and/or 49 and/or 50 and/or 51 and/or 52 and/or 53 and/or 54 and/or 55 and/or 56 and/or 57 and/or 58 and/or 59 and/or 60 and/or 61 and/or 62 and/or 63 and/or 64 and/or 65 and/or 66 and/or 67 and/or 68 and/or 69 and/or 70 and/or 71 and/or 72 and/or 73 and/or 74 and/or 75 and/or 76 and/or 77 and/or 78 and/or 79 and/or 80 and/or 81 and/or 82 and/or 83 and/or 84 and/or 85 and/or 86 and/or 87 and/or 88 and/or 89 and/or 90 and/or 91 and/or 92 and/or 93 and/or 94 and/or 95 and/or 96 and/or 97 and/or 98 and/or 99 and/or 100 and/or 101 and/or 102 and/or 103 and/or 104 and/or 105 and/or 106 and/or 107 and/or 108 and/or 109 and/or 110 and/or 111 and/or 112 and/or 113 and/or 114 and/or 115 and/or 116 and/or 117 in SEQ ID NO: 11.

However, since it is not expected that immunization against the pre-propeptide form of ghrelin would be of any relevance compared to immunization against the propeptide, it is preferred that the introduction is performed after any one of amino acids 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, and 117 in SEQ ID NO: 11 (since amino acid 23 is the last amino acid in the signal sequence in human ghrelin, so that the introduction is performed in the propeptide region of the ghrelin molecule) and that substantially no amino acids from the signal sequence is part of the immunogen.

In embodiments where it is desired to target the complete propeptide, cf. above, it is preferred to avoid destruction of B-cell epitopes in proghrelin – therefore, introduction of the foreign T_H epitopes should in this embodiment be accompanied by no or only very limited deletions (deletions that do not destroy B-cell epitopes) of any one of amino acids 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, and 117 in SEQ ID NO: 11.

If, on the other hand, it is desired to provide an "immunogenized" ghrelin that does not include the sequence of mature ghrelin, cf. above, the introduction will preferably include deletion of a substantial number of amino acids 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, and 51 in SEQ ID NO: 11. Consequently, it is preferred that only non-destructive (*i.e.* B-cell epitope conserving) deletions/substitutions are made among amino acids 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, and 117 in SEQ ID NO: 11. In this embodiment it is most preferred that all of amino acids 1-51 are deleted.

Finally, if it is desired to provide a ghrelin variant that does not include any B-cell epitopes of the part of proghrelin that does not form part of the mature molecule, then the introduction of the foreign T_H epitope should be accompanied by deletion of a substantial number of amino acids 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, and 117 in SEQ ID NO: 11. Consequently, it is preferred that only non-destructive (*i.e.*

B-cell epitope conserving) deletions/substitutions) are made among amino acids 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, and 51 in SEQ ID NO: 11. It is in this embodiment most preferred that all of amino acids 52-117 are deleted.

- 5 Another embodiment of the present invention is the presentation of the ghrelin analogues which do not include any subsequence of ghrelin that binds productively to MHC class II molecules initiating a T-cell response.

The rationale behind such a strategy for design of the immunogen that engages the immune system to induce an anti-ghrelin immune response is the following: It has been noted that
10 when immunizing with autologous proteins formulated in an adjuvant which is sufficiently strong to break the body's tolerance towards the autologous protein, there is a danger that in some vaccinated individuals the immune response induced cannot be discontinued simply by discontinuing the immunisation. This is because the induced immune response in such individuals is most likely driven by a *native* T_H epitope of the autologous protein, and this has the
15 adverse effect that the vaccinated individual's own protein will be able to function as an immunizing agent in its own right: An autoimmune condition has thus been established.

The preferred methods including use of foreign T_H epitopes have to the best of the inventors' knowledge never been observed to produce this effect, because the anti-self immune response is driven by a *foreign* T_H epitope, and it has been repeatedly demonstrated by the in-
20 ventors that the induced immune response invoked by the preferred technology indeed declines after discontinuation of immunizations. However, in theory it could happen in a few individuals that the immune response would also be driven by an autologous T_H epitope of the relevant self-protein one immunises against) – this is especially relevant when considering self-proteins that are relatively abundant, whereas other therapeutically relevant self-proteins
25 are only present locally or in so low amounts in the body, that a "self-immunization effect" is not a possibility; however, for ghrelin, this effect cannot be excluded.

One very simple way of avoiding this self-immunisation is hence to altogether avoid inclusion in the immunogen of peptide sequences that *could* serve as T_H epitopes (and since peptides shorter than about 9 amino acids cannot serve as T_H epitopes, the use of shorter fragments is
30 one simple and feasible approach). Therefore, this embodiment of the invention also serves to ensure that the immunogen does not include peptide sequences of the target ghrelin that could serve as "self-stimulating T_H epitopes" including sequences that merely contain conservative substitutions in a sequence of the target protein that might otherwise function as a T_H epitope.

Preferred embodiments of the immune system presentation of the analogues of ghrelin involve the use of a chimeric peptide comprising at least one ghrelin derived peptide, which does not bind productively to MHC class II molecules, and at least one foreign T-helper epitope. Moreover, it is preferred that the ghrelin derived peptide harbours a B-cell epitope. It is especially advantageous if the immunogenic analogue is one, wherein the amino acid sequences comprising one or more B-cell epitopes are represented either as a continuous sequence or as a sequence including inserts, wherein the inserts comprise foreign T-helper epitopes.

Again, such an embodiment is most preferred when the suitable B-cell epitope carrying regions of ghrelin are constituted by short peptide stretches that in no way would be able to bind productively to an MHC Class II molecule. The selected B-cell epitope or -epitopes of ghrelin should therefore comprise at most 9 consecutive amino acids of ghrelin of a relevant animal, that is, at least 9 consecutive amino acids in e.g. SEQ ID NO: 9, 10, 11, 12, 13, or 14. Shorter peptides are preferred, such as those having at most 8, 7, 6, 5, 4, or 3 consecutive amino acids from the ghrelin amino acid sequence.

It is preferred that the analogue comprises at least one subsequence of SEQ ID NO: 9, 10, 11, 12, 13, or 14 so that each such at least one subsequence independently consists of amino acid stretches from ghrelin selected from the group consisting of 9 consecutive amino acids, 8 consecutive amino acids, 7 consecutive amino acids, 6 consecutive amino acids, 5 consecutive amino acids, 4 consecutive amino acids, and 3 consecutive amino acids.

It is especially preferred that the consecutive amino acids begins at an amino acid residue selected from the group consisting of residue 1 and/or 2 and/or 3 and/or 4 and/or 5 and/or 6 and/or 7 and/or 8 and/or 9 and/or 10 and/or 11 and/or 12 and/or 13 and/or 14 and/or 15 and/or 16 and/or 17 and/or 18 and/or 19 and/or 20 and/or 21 and/or 22 and/or 23 and/or 24 and/or 25 and/or 26 and/or 27 and/or 28 and/or 29 and/or 30 and/or 31 and/or 32 and/or 33 and/or 34 and/or 35 and/or 36 and/or 37 and/or 38 and/or 39 and/or 40 and/or 41 and/or 42 and/or 43 and/or 44 and/or 45 and/or 46 and/or 47 and/or 48 and/or 49 and/or 50 and/or 51 and/or 52 and/or 53 and/or 54 and/or 55 and/or 56 and/or 57 and/or 58 and/or 59 and/or 60 and/or 61 and/or 62 and/or 63 and/or 64 and/or 65 and/or 66 and/or 67 and/or 68 and/or 69 and/or 70 and/or 71 and/or 72 and/or 73 and/or 74 and/or 75 and/or 76 and/or 77 and/or 78 and/or 79 and/or 80 and/or 81 and/or 82 and/or 83 and/or 84 and/or 85 and/or 86 and/or 87 and/or 88 and/or 89 and/or 90 and/or 91 and/or 92 and/or 93 and/or 94 and/or 95 and/or 96 and/or 97 and/or 98 and/or 99 and/or 100 and/or 101 and/or 102 and/or 103 and/or 104 and/or 105 and/or 106 and/or 107 and/or 108 and/or 109 and/or 110 and/or 111 and/or 112 and/or 113 and/or 114 and/or 115 and/or 116 in SEQ ID NO: 9, 10, 11, 12, 13, or 14, where this is possible given the length of the consecutive stretch and the relevant ghrelin polypeptide.

In all variants described above where the n-octanylated serine of mature ghrelin could be present, it is preferred to prepare the immunogenic construct in such a way that the n-octanylation is absent (either by preparing the constructs by means of peptide synthesis or by using an expression system that will not introduce the n-octanylation). In this way it is ensured that the constructs are not physiologically active in the CNS.

Formulation of ghrelin and modified ghrelin polypeptides

When effecting presentation of the ghrelin polypeptide or the modified ghrelin polypeptide to an animal's immune system by means of administration thereof to the animal, the formulation of the polypeptide follows the principles generally acknowledged in the art.

- 10 Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines; cf. the detailed discussion of adjuvants below.

- The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously, intracutaneously, intradermally, subdermally or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral, buccal, sublingual, intraperitoneal, intravaginal, anal, epidural, spinal, and intracranial formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%. For oral formulations, cholera toxin is an interesting formulation partner (and also a possible conjugation partner).

The polypeptides may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts
5 formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in
10 such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, *e.g.*, the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 µg to 2,000 µg (even though higher amounts
15 in the 1-10 mg range are contemplated), such as in the range from about 0.5 µg to 2,000 µg or 0.5 µg to 1,000 µg, preferably in the range from 1 µg to 500 µg and especially in the range from about 10 µg to 100 µg. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

20 The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated and the formulation of the antigen.

25 Some of the polypeptides of the vaccine are sufficiently immunogenic in a vaccine, but for some of the others the immune response will be enhanced if the vaccine further comprises an adjuvant substance.

Various methods of achieving adjuvant effect for the vaccine are known. General principles and methods are detailed in "The Theory and Practical Application of Adjuvants", 1995, Duncan E.S. Stewart-Tull (ed.), John Wiley & Sons Ltd, ISBN 0-471-95170-6, and also in "Vaccines: New Generation Immunological Adjuvants", 1995, Gregoriadis G *et al.* (eds.), Plenum Press, New York, ISBN 0-306-45283-9, both of which are hereby incorporated by reference
30 herein.

It is especially preferred to use an adjuvant which can be demonstrated to facilitate breaking
35 of the autotolerance to autoantigens; in fact, this is essential in cases where unmodified ghere-

lin is used as the active ingredient in the autovaccine. Non-limiting examples of suitable adjuvants are selected from the group consisting of an immune targeting adjuvant; an immune modulating adjuvant such as a toxin, a cytokine, and a mycobacterial derivative; an oil formulation; a polymer; a micelle forming adjuvant; a saponin; an immunostimulating complex matrix (ISCOM matrix); a particle; DDA; aluminium adjuvants; DNA adjuvants; γ -inulin; and an encapsulating adjuvant. In general it should be noted that the disclosures above which relate to compounds and agents useful as first, second and third moieties in the analogues also refer *mutatis mutandis* to their use in the adjuvant of a vaccine of the invention.

The application of adjuvants include use of agents such as aluminium hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in buffered saline, admixture with synthetic polymers of sugars (e.g. Carbopol®) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C for 30 second to 2 minute periods respectively and also aggregation by means of cross-linking agents are possible. Aggregation by reactivation with pepsin treated antibodies (Fab fragments) to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. Admixture with oils such as squalene and IFA is also preferred.

According to the invention DDA (dimethyldioctadecylammonium bromide) is an interesting candidate for an adjuvant as is DNA and γ -inulin, but also Freund's complete and incomplete adjuvants as well as *quillaja* saponins such as QuilA and QS21 are interesting as is RIBI. Further possibilities are monophosphoryl lipid A (MPL), the above mentioned C3 and C3d, and muramyl dipeptide (MDP).

Liposome formulations are also known to confer adjuvant effects, and therefore liposome adjuvants are preferred according to the invention.

Also immunostimulating complex matrix type (ISCOM® matrix) adjuvants are preferred choices according to the invention, especially since it has been shown that this type of adjuvants are capable of up-regulating MHC Class II expression by APCs. An ISCOM® matrix consists of (optionally fractionated) saponins (triterpenoids) from *Quillaja saponaria*, cholesterol, and phospholipid. When admixed with the immunogenic protein, the resulting particulate formulation is what is known as an ISCOM particle where the saponin constitutes 60-70% w/w, the cholesterol and phospholipid 10-15% w/w, and the protein 10-15% w/w. Details relating to composition and use of immunostimulating complexes can e.g. be found in the above-mentioned text-books dealing with adjuvants, but also Morein B *et al.*, 1995, Clin. Immunother. **3**: 461-475 as well as Barr IG and Mitchell GF, 1996, Immunol. and Cell Biol. **74**: 8-25

(both incorporated by reference herein) provide useful instructions for the preparation of complete immunostimulating complexes.

Another highly interesting (and thus, preferred) possibility of achieving adjuvant effect is to employ the technique described in Gosselin *et al.*, 1992 (which is hereby incorporated by reference herein). In brief, the presentation of a relevant antigen such as an antigen of the present invention can be enhanced by conjugating the antigen to antibodies (or antigen binding antibody fragments) against the Fc γ receptors on monocytes/macrophages. Especially conjugates between antigen and anti-Fc γ RI have been demonstrated to enhance immunogenicity for the purposes of vaccination.

- 10 Other possibilities involve the use of the targeting and immune modulating substances (*i.a.* cytokines) mentioned above as candidates for the first and second moieties in the modified versions of ghrelin. In this connection, also synthetic inducers of cytokines like poly I:C are possibilities.

- 15 Suitable mycobacterial derivatives are selected from the group consisting of muramyl dipeptide, complete Freund's adjuvant, RIBI, and a diester of trehalose such as TDM and TDE.

Suitable immune targeting adjuvants are selected from the group consisting of CD40 ligand and CD40 antibodies or specifically binding fragments thereof (*cf.* the discussion above), mannose, a Fab fragment, and CTLA-4.

- 20 Suitable polymer adjuvants are selected from the group consisting of a carbohydrate such as dextran, PEG, starch, mannan, and mannose; a plastic polymer such as; and latex such as latex beads.

- Yet another interesting way of modulating an immune response is to include the ghrelin immunogen (optionally together with adjuvants and pharmaceutically acceptable carriers and vehicles) in a "virtual lymph node" (VLN) (a proprietary medical device developed by Immunotherapy, Inc., 360 Lexington Avenue, New York, NY 10017-6501). The VLN (a thin tubular device) mimics the structure and function of a lymph node. Insertion of a VLN under the skin creates a site of sterile inflammation with an upsurge of cytokines and chemokines. T- and B-cells as well as APCs rapidly respond to the danger signals, home to the inflamed site and accumulate inside the porous matrix of the VLN. It has been shown that the necessary antigen dose required to mount an immune response to an antigen is reduced when using the VLN and that immune protection conferred by vaccination using a VLN surpassed conventional immunization using Ribi as an adjuvant. The technology is *i.a.* described briefly in Gelber C *et al.*, 1998, "Elicitation of Robust Cellular and Humoral Immune Responses to Small Amounts of Immunogens Using a Novel Medical Device Designated the Virtual Lymph Node", in: "From
- 25
30

the Laboratory to the Clinic, Book of Abstracts, October 12th - 15th 1998, Seascape Resort, Aptos, California".

Microparticle formulation of vaccines has been shown in many cases to increase the immunogenicity of protein antigens and is therefore another preferred embodiment of the invention.

- 5 Microparticles are made either as co-formulations of antigen with a polymer, a lipid, a carbohydrate or other molecules suitable for making the particles or the microparticles can be homogeneous particles consisting of only the antigen itself.

- 10 Examples of polymer based microparticles are PLGA and PVP based particles (Gupta RK *et al.*, 1998) where the polymer and the antigen are condensed into a solid particle. Lipid based particles can be made as micelles of the lipid (so-called liposomes) entrapping the antigen within the micelle (Pietrobon PJ, 1995). Carbohydrate based particles are typically made of a suitable degradable carbohydrate such as starch or chitosan. The carbohydrate and the antigen are mixed and condensed into particles in a process similar to the one used for polymer particles (Kas HS *et al.*, 1997).

- 15 Particles consisting only of the antigen can be made by various spraying and freeze-drying techniques. Especially suited for the purposes of the present invention is the super critical fluid technology that is used to make very uniform particles of controlled size (York P, 1999 & Shekunov B *et al.*, 1999).

- 20 It is expected that the vaccine should be administered at least once a year, such as at least 1, 2, 3, 4, 5, 6, and 12 times a year. More specifically, 1-12 times per year is expected, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 times a year to an individual in need thereof. It has previously been shown that the memory immunity induced by the use of the preferred auto-vaccines according to the invention is not permanent, and therefore the immune system needs to be periodically challenged with the analogues.

- 25 Due to genetic variation, different individuals may react with immune responses of varying strength to the same polypeptide. Therefore, the vaccine according to the invention may comprise several different polypeptides in order to increase the immune response, cf. also the discussion above concerning the choice of foreign T-cell epitope introductions. The vaccine may comprise two or more polypeptides, where all of the polypeptides are as defined above.

- 30 The vaccine may consequently comprise 3-20 different modified or unmodified polypeptides, such as 3-10 different polypeptides. However, normally the number of polypeptides will be sought kept to a minimum such as 1 or 2 polypeptides.

As an alternative to the ghrelin analogues of the invention, it is also possible to immunize by using anti-idiotypic antibodies or even mimotopes. The technologies for preparing anti-idiotypic antibodies that mimic a ghrelin epitope are known in the art, but one especially interesting version involves use of autologous anti-idiotypic antibodies, which are reactive with an anti-ghrelin antibody and which are modified by introduction of a foreign T helper epitope as generally described herein. Mimotopes can be isolated from libraries of random peptides that are screened in phage display against antibodies that bind ghrelin specifically.

Nucleic acid vaccination

As an alternative to classic administration of a peptide-based vaccine, the technology of nucleic acid vaccination (also known as "nucleic acid immunisation", "genetic immunisation", and "gene immunisation") offers a number of attractive features.

First, in contrast to the traditional vaccine approach, nucleic acid vaccination does not require resource consuming large-scale production of the immunogenic agent (e.g. in the form of industrial scale fermentation of microorganisms producing modified ghrelin). Furthermore, there is no need to devise purification and refolding schemes for the immunogen. And finally, since nucleic acid vaccination relies on the biochemical apparatus of the vaccinated individual in order to produce the expression product of the nucleic acid introduced, the optimum post-translational processing of the expression product is expected to occur; this is especially important in the case of autovaccination, since, as mentioned above, a significant fraction of the original ghrelin B-cell epitopes should be preserved in the modified molecule, and since B-cell epitopes in principle can be constituted by parts of any (bio)molecule (e.g. carbohydrate, lipid, protein etc.). Therefore, native glycosylation and lipidation patterns of the immunogen may very well be of importance for the overall immunogenicity and this is expected to be ensured by having the host producing the immunogen.

Hence, a preferred embodiment of the invention comprises effecting presentation of modified ghrelin to the immune system by introducing nucleic acid(s) encoding the modified ghrelin into the animal's cells and thereby obtaining *in vivo* expression by the cells of the nucleic acid(s) introduced.

In this embodiment, the introduced nucleic acid is preferably DNA which can be in the form of naked DNA, DNA formulated with charged or uncharged lipids, DNA formulated in liposomes, DNA included in a viral vector, DNA formulated with a transfection-facilitating protein or polypeptide, DNA formulated with a targeting protein or polypeptide, DNA formulated with calcium precipitating agents, DNA coupled to an inert carrier molecule, DNA encapsulated in a polymer, e.g. in PLGA (cf. the microencapsulation technology described in WO 98/31398) or in chitin or chitosan, and DNA formulated with an adjuvant. In this context it is noted that

practically all considerations pertaining to the use of adjuvants in traditional vaccine formulation apply for the formulation of DNA vaccines. Hence, all disclosures herein which relate to use of adjuvants in the context of polypeptide based vaccines apply *mutatis mutandis* to their use in nucleic acid vaccination technology.

- 5 As for routes of administration and administration schemes of polypeptide based vaccines which have been detailed above, these are also applicable for the nucleic acid vaccines of the invention and all discussions above pertaining to routes of administration and administration schemes for polypeptides apply *mutatis mutandis* to nucleic acids. To this should be added that nucleic acid vaccines can suitably be administered intravenously and intraarterially.
- 10 Furthermore, it is well-known in the art that nucleic acid vaccines can be administered by use of a so-called gene gun, and hence also this and equivalent modes of administration are regarded as part of the present invention. Finally, also the use of a VLN in the administration of nucleic acids has been reported to yield good results, and therefore this particular mode of administration is particularly preferred.
- 15 Furthermore, the nucleic acid(s) used as an immunization agent can contain regions encoding the 1st, 2nd and/or 3rd moieties, e.g. in the form of the immunomodulating substances described above such as the cytokines discussed as useful adjuvants. A preferred version of this embodiment encompasses having the coding region for the analogue and the coding region for the immunomodulator in different reading frames or at least under the control of different
- 20 promoters. Thereby it is avoided that the analogue or epitope is produced as a fusion partner to the immunomodulator. Alternatively, two distinct nucleotide fragments can be used, but this is less preferred because of the advantage of ensured co-expression when having both coding regions included in the same molecule.

Accordingly, the invention also relates to a composition for inducing production of antibodies

- 25 against ghrelin, the composition comprising

- a nucleic acid fragment or a vector of the invention (cf. the discussion of vectors below), and
- a pharmaceutically and immunologically acceptable vehicle and/or carrier and/or adjuvant as discussed above.

- 30 Under normal circumstances, the ghrelin variant-encoding nucleic acid is introduced in the form of a vector wherein expression is under control of a viral promoter. For more detailed discussions of vectors and DNA fragments according to the invention, cf. the discussion below. Also, detailed disclosures relating to the formulation and use of nucleic acid vaccines are available, cf. Donnelly JJ et al, 1997, Annu. Rev. Immunol. **15**: 617-648 and Donnelly JJ et

al., 1997, Life Sciences **60**: 163-172. Both of these references are incorporated by reference herein.

Live vaccines

A third alternative for effecting presentation of modified ghrelin to the immune system is the use of live vaccine technology. In live vaccination, presentation to the immune system is effected by administering, to the animal, a non-pathogenic microorganism which has been transformed with a nucleic acid fragment encoding a modified ghrelin or with a vector incorporating such a nucleic acid fragment. The non-pathogenic microorganism can be any suitable attenuated bacterial strain (attenuated by means of passaging or by means of removal of pathogenic expression products by recombinant DNA technology), e.g. *Mycobacterium bovis* BCG., non-pathogenic *Streptococcus* spp., *E. coli*, *Salmonella* spp., *Vibrio cholerae*, *Shigella*, etc. Reviews dealing with preparation of state-of-the-art live vaccines can e.g. be found in Saliou P, 1995, Rev. Prat. **45**: 1492-1496 and Walker PD, 1992, Vaccine **10**: 977-990, both incorporated by reference herein. For details about the nucleic acid fragments and vectors used in such live vaccines, cf. the discussion below.

As an alternative to bacterial live vaccines, the nucleic acid fragment of the invention discussed below can be incorporated in a non-virulent viral vaccine vector such as a vaccinia strain or any other suitable pox virus.

Normally, the non-pathogenic microorganism or virus is administered only once to the animal, but in certain cases it may be necessary to administer the microorganism more than once in a lifetime in order to maintain protective immunity. It is even contemplated that immunization schemes as those detailed above for polypeptide vaccination will be useful when using live or virus vaccines.

Alternatively, live or virus vaccination is combined with previous or subsequent polypeptide and/or nucleic acid vaccination. For instance, it is possible to effect primary immunization with a live or virus vaccine followed by subsequent booster immunizations using the polypeptide or nucleic acid approach.

The microorganism or virus can be transformed with nucleic acid(s) containing regions encoding the 1st, 2nd and/or 3rd moieties, e.g. in the form of the immunomodulating substances described above such as the cytokines discussed as useful adjuvants. A preferred version of this embodiment encompasses having the coding region for the analogue and the coding region for the immunomodulator in different reading frames or at least under the control of different promoters. Thereby it is avoided that the analogue or epitopes are produced as fusion partners to the immunomodulator. Alternatively, two distinct nucleotide fragments can be

used as transforming agents. Of course, having the 1st and/or 2nd and/or 3rd moieties in the same reading frame can provide as an expression product, an analogue of the invention, and such an embodiment is especially preferred according to the present invention.

Use of the method of the invention in disease treatment and other settings

5 As will be appreciated from above, vaccination against ghrelin is expected to provide an effective means for reducing excess body fat in individuals in need thereof. Furthermore, it has been shown that co-expression of the ghrelin receptor (GSH-R) and ghrelin does occur in prostate cancer cells (Jeffery PL *et al.*, 2002, J Endocrinol, 172(3): R7-11). Expression of GSG-R has also been reported in some endocrine tumours (Volante M *et al.*, 2002 J Clin En-
10 docrinol Metab, 87(3): 1300-8), and Papotti M *et al.* (2001, J Clin Endocrinol Metab, 86(10): 5052-9) report that the majority (75%) of gastric carcinoids and 25% of intestinal endocrine tumours were immunoreactive for ghrelin. In other words, the methods of the invention may be practiced as a treatment of obesity and of ghrelin and ghrelin-receptor related cancers.

It must be noted that very low amounts of circulating ghrelin may result in a loss of interest
15 in food – the individual having such a low ghrelin concentration does not have a drive to eat when necessary. It is thus contemplated that the presently suggested immunotherapeutic treatment of humans should be accompanied by a controlled diet in order to ensure that the person undergoing treatment ingests necessary nutrients. At the same time the rate of weight loss should be carefully monitored in order to avoid too drastic reductions in body weight over
20 time and it should be ensured that the treated subject exerts a physical behaviour that aims at preserving muscle mass etc.

In the Examples are reported experiments with a few immunogenic variants of rat ghrelin which was used as immunogens for inducing an immune response against autologous ghrelin in rats. As expected, the immunizations resulted in induction in the rats of high-titered anti-
25 bodies reactive with the autologous rat ghrelin. Surprisingly, however, these high antibody titers were accompanied by an *increase* in the levels of circulating ghrelin in these rats, and this also correlated with an increase in body weight in all rats tested. Whether the body weight increase was due to increase in muscle mass, body fat or both is not known yet.

It is not expected that these effects constitute a general phenomenon and there are several
30 explanations that can account (alone or in any combination) for the increase in serum level of ghrelin in response to the immunization:

- the existence of a negative feed-back loop that naturally regulates ghrelin production in response to the concentration of molecules which are up-regulated by ghrelin,
- a direct effect of some of the induced anti-ghrelin antibodies on a receptor that competes

with ghrelin for binding to a regulatory molecule,

- activation by some of the induced anti-ghrelin antibodies of an effector molecule which is normally activated by a ghrelin binding protein thereby stimulating ghrelin production,
- an up-concentration of total serum ghrelin in the blood-stream because of the presence of the ghrelin binding antibodies (i.e. a compartmentalization of ghrelin) which leads to a larger pool of accessible ghrelin and possibly also to a prolonged serum half-life of ghrelin,
- blocking effects by some of the antibodies on the interaction between ghrelin and a "sensor receptor" or "decoy receptor" involved in the control of ghrelin expression etc.

None of these possible explanations can be ruled out based on the present experiments. In particular, it is noteworthy that all constructs tested in the Examples include the complete sequence of mature rat ghrelin, meaning that antibodies against all possible ghrelin B-cell epitopes must be expected to be present in the sera from the immunized rats. And, if for instance the apparent up-regulation in ghrelin is a consequence of antibodies binding a site that interacts with a ghrelin binding molecule or receptor, then a number of the ghrelin constructs disclosed herein will not lead to induction of antibodies having the stimulating effect on ghrelin production and thereby these constructs will lead to the initially expected down-regulation of ghrelin.

Alternatively, if the effect is simply a consequence of an expansion of the total available amount of ghrelin in serum and a prolongation of serum half-life, this opens for a completely new therapeutic approach when using vaccination against autologous proteins and other molecules. For example, some hormones like ghrelin need to cross a barrier which is not permeable for antibodies before they can exert their effect in the target tissue. When immunizing actively against such molecules, the serum level of the molecule (antibody-bound and free) and their half-life will increase, thereby leading to a larger pool of accessible molecule. Due to the equilibrium state between receptor bound molecule in the target organs and the total pool in serum, the effect will paradoxically be a net increase in stimulation exerted by the molecule because larger amounts of the molecule can traverse the antibody-impermeable barrier. Hence, the presence of the antibodies binding the molecule leads to an increase in the effect exerted by the molecule. This concept is believed to be novel, and can also be applied using other technologies where the serum pool of a particular molecule can be increased (administration of soluble receptors binding the molecule, administration of monoclonal antibodies binding the molecule etc.).

At any rate, these particular findings have identified an unexpected use of immunization against ghrelin, namely in conditions where an increased level of ghrelin is of interest. Conditions such as anorexia and cachexia have been previously suggested as indications for ghrelin therapy as have cardiovascular diseases such as heart failure. Such diseases and conditions

are all interesting in embodiments of the present invention where induction of an anti-ghrelin immune response leads to an increase in circulating ghrelin levels.

Further, it is also of interest to utilise this unexpected aspect in the farm industry. It is of value to increase the production of fat in milk producing cows and in a number of other animals, where fat deposits and fat production are relevant for the taste and quality of the farm product in question.

Also, ghrelin is known to be a stimulator of the discharge of growth hormone, so in conditions or situations where the effects of an increase in growth hormone production is of interest, the present method affords an alternative, where repeated administrations of growth hormone can be avoided. Such applications involve the use for in vitro fertilization (where GH treatment has been suggested as adjuvating therapy), the use in wound healing (e.g. after severe burns) and generally in treatment of severe trauma, as mentioned above for inducing growth in farm animals, for prolonging survival in patients with heart failure, and the use as an "anti-aging" drug.

15 Peptides, polypeptides, and compositions of the invention

As will be apparent from the above, the present invention is based on the concept of immunising individuals against the ghrelin antigen. The preferred way of obtaining such an immunization is to use modified versions of ghrelin, thereby providing molecules which have not previously been disclosed in the art.

20 It is believed that the modified ghrelin molecules discussed herein are inventive in their own right, and therefore an important part of the invention pertains to a ghrelin analogue which is derived from an animal ghrelin wherein is introduced a modification which has as a result that immunization of the animal with the analogue induces production of antibodies cross-reacting with the unmodified ghrelin polypeptide. Preferably, the nature of the modification conforms with the types of modifications described above when discussing various embodiments of the method of the invention when using modified ghrelin. Hence, any disclosure presented herein pertaining to modified ghrelin molecules are relevant for the purpose of describing the ghrelin analogues of the invention, and any such disclosures apply *mutatis mutandis* to the description of these analogues.

30 It should be noted that preferred modified ghrelin molecules comprise modifications which results in a polypeptide having a sequence identity of at least 70% with ghrelin or with a sub-sequence thereof of at least 10 amino acids in length. Higher sequence identities are preferred, e.g. at least 75% or even at least 80% or 85%. The sequence identity for proteins and nucleic acids can be calculated as $(N_{ref} - N_{dif}) \cdot 100 / N_{ref}$, wherein N_{dif} is the total number of non-

identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC ($N_{diff}=2$ and $N_{ref}=8$).

The invention also pertains to compositions useful in exercising the method of the invention.

- 5 Hence, the invention also relates to an immunogenic composition comprising an immunogenically effective amount of a ghrelin polypeptide which is a self-protein in an animal or a subsequence of such a ghrelin polypeptide, said ghrelin polypeptide or subsequence being formulated together with an immunologically acceptable adjuvant so as to break the animal's autotolerance towards the ghrelin polypeptide, the composition further comprising a pharmaceutical and immunologically acceptable vehicle and/or carrier. In other words, this part of the invention pertains to the formulations of naturally occurring ghrelin polypeptides/subsequences which have been described in connection with embodiments of the method of the invention.

- 15 The invention also relates to an immunogenic composition comprising an immunologically effective amount of a ghrelin analogue defined above, said composition further comprising a pharmaceutically and immunologically acceptable diluent and/or vehicle and/or carrier and/or excipient and optionally an adjuvant. In other words, this part of the invention concerns formulations of modified ghrelin, essentially as described hereinabove. The choice of adjuvants, carriers, and vehicles is accordingly in line with what has been discussed above when referring to formulation of modified and unmodified ghrelin for use in the inventive method for the immunizing against autologous ghrelin.

- 25 The polypeptides are prepared according to methods well-known in the art. Longer polypeptides are normally prepared by means of recombinant gene technology including introduction of a nucleic acid sequence encoding the ghrelin analogue into a suitable vector, transformation of a suitable host cell with the vector, expression of the nucleic acid sequence, recovery of the expression product from the host cells or their culture supernatant, and subsequent purification and optional further modification, e.g. refolding or derivatization.

- 30 Shorter peptides are preferably prepared by means of the well-known techniques of solid- or liquid-phase peptide synthesis. However, recent advances in this technology has rendered possible the production of full-length polypeptides and proteins by these means, and therefore it is also within the scope of the present invention to prepare the long constructs by synthetic means.

Nucleic acid fragments and vectors of the invention

It will be appreciated from the above disclosure that modified ghrelin polypeptides can be prepared by means of recombinant gene technology but also by means of chemical synthesis or semisynthesis; the latter two options are especially relevant when the modification consists
5 in coupling to protein carriers (such as KLH, diphtheria toxoid, tetanus toxoid, and BSA) and non-proteinaceous molecules such as carbohydrate polymers and of course also when the modification comprises addition of side chains or side groups to a ghrelin polypeptide-derived peptide chain.

For the purpose of recombinant gene technology, and of course also for the purpose of nucleic
10 acid immunization, nucleic acid fragments encoding modified ghrelin are important chemical products. Hence, an important part of the invention pertains to a nucleic acid fragment which encodes a ghrelin analogue, *i.e.* a ghrelin derived polypeptide which either comprises the natural ghrelin sequence to which has been added or inserted a fusion partner or, preferably a ghrelin derived polypeptide wherein has been introduced a foreign T-cell epitope by means of
15 insertion and/or addition, preferably by means of substitution and/or deletion. The nucleic acid fragments of the invention are either DNA or RNA fragments.

The nucleic acid fragments of the invention will normally be inserted in suitable vectors to form cloning or expression vectors carrying the nucleic acid fragments of the invention; such novel vectors are also part of the invention. Details concerning the construction of these vec-
20 tors of the invention will be discussed in context of transformed cells and microorganisms below. The vectors can, depending on purpose and type of application, be in the form of plasmids, phages, cosmids, mini-chromosomes, or virus, but also naked DNA which is only expressed transiently in certain cells is an important vector. Preferred cloning and expression vectors of the invention are capable of autonomous replication, thereby enabling high copy-
25 numbers for the purposes of high-level expression or high-level replication for subsequent cloning.

The general outline of a vector of the invention comprises the following features in the 5'→3' direction and in operable linkage: a promoter for driving expression of the nucleic acid fragment of the invention, optionally a nucleic acid sequence encoding a leader peptide enabling
30 secretion (to the extracellular phase or, where applicable, into the periplasma) of or integration into the membrane of the polypeptide fragment, the nucleic acid fragment of the invention, and optionally a nucleic acid sequence encoding a terminator. When operating with expression vectors in producer strains or cell-lines it is for the purposes of genetic stability of the transformed cell preferred that the vector when introduced into a host cell is integrated in
35 the host cell genome. In contrast, when working with vectors to be used for effecting *in vivo* expression in an animal (*i.e.* when using the vector in DNA vaccination) it is for security rea-

sons preferred that the vector is not incapable of being integrated in the host cell genome; typically, naked DNA or non-integrating viral vectors are used, the choices of which are well-known to the person skilled in the art.

The vectors of the invention are used to transform host cells to produce the modified ghrelin polypeptide of the invention. Such transformed cells, which are also part of the invention, can be cultured cells or cell lines used for propagation of the nucleic acid fragments and vectors of the invention, or used for recombinant production of the modified ghrelin polypeptides of the invention. Alternatively, the transformed cells can be suitable live vaccine strains wherein the nucleic acid fragment (one single or multiple copies) have been inserted so as to effect secretion or integration into the bacterial membrane or cell-wall of the modified ghrelin.

Preferred transformed cells of the invention are microorganisms such as bacteria (such as the species *Escherichia* [e.g. *E. coli*], *Bacillus* [e.g. *Bacillus subtilis*], *Salmonella*, or *Mycobacterium* [preferably non-pathogenic, e.g. *M. bovis* BCG]), yeasts (such as *Saccharomyces cerevisiae*), and protozoans. Alternatively, the transformed cells are derived from a multicellular organism such as a fungus, an insect cell, a plant cell, or a mammalian cell. Most preferred are cells derived from a human being, cf. the discussion of cell lines and vectors below. Recent results have shown great promise in the use of a commercially available *Drosophila melanogaster* cell line (the Schneider 2 (S₂) cell line and vector system available from Invitrogen) for the recombinant production of ghrelin analogues of the invention, and therefore this expression system is particularly preferred. Also the *spodoptera* cells (SF cells) SF9 and SF21 are preferred.

For the purposes of cloning and/or optimised expression it is preferred that the transformed cell is capable of replicating the nucleic acid fragment of the invention. Cells expressing the nucleic acid fragment are preferred useful embodiments of the invention; they can be used for small-scale or large-scale preparation of the modified ghrelin or, in the case of non-pathogenic bacteria, as vaccine constituents in a live vaccine.

When producing the modified ghrelin of the invention by means of transformed cells, it is convenient, although far from essential, that the expression product is either exported out into the culture medium or carried on the surface of the transformed cell.

When an effective producer cell has been identified it is preferred, on the basis thereof, to establish a stable cell line which carries the vector of the invention and which expresses the nucleic acid fragment encoding the modified ghrelin. Preferably, this stable cell line secretes or carries the ghrelin analogue of the invention, thereby facilitating purification thereof.

- In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with the hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using
- 5 pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar *et al.*, 1977). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the prokaryotic microorganism for expression.
- 10 Those promoters most commonly used in prokaryotic recombinant DNA construction include the B-lactamase (penicillinase) and lactose promoter systems (Chang *et al.*, 1978; Itakura *et al.*, 1977; Goeddel *et al.*, 1979) and a tryptophan (*trp*) promoter system (Goeddel *et al.*, 1979; EP-A-0 036 776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have
- 15 been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist *et al.*, 1980). Certain genes from prokaryotes may be expressed efficiently in *E. coli* from their own promoter sequences, precluding the need for addition of another promoter by artificial means.

- In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used, and
- 20 here the promoter should be capable of driving expression. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb *et al.*, 1979; Kingsman *et al.*, 1979; Tschemper *et al.*, 1980). This plasmid already contains the *trp1* gene which provides a
- 25 selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the *trp1* lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

- Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate
- 30 kinase (Hitzman *et al.*, 1980) or other glycolytic enzymes (Hess *et al.*, 1968; Holland *et al.*, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated
- 35 with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, Isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 293, *Spodoptera frugiperda* (SF) cells (commercially available as complete expression systems from *i.a.* Protein Sciences, 1000 Research Parkway, Meriden, CT 06450, U.S.A. and from Invitrogen), and MDCK cell lines. In the present invention, an especially preferred cell line is S₂ available from Invitrogen, PO Box 2312, 9704 CH Groningen, The Netherlands.

Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Flers *et al.*, 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the *HindIII* site toward the *BglII* site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (*e.g.*, Polyoma, Adeno, VSV, BPV) or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Identification of useful ghrelin analogues

It will be clear to the skilled person that not all variants or modifications of native ghrelin will have the ability to elicit antibodies in an animal which are cross-reactive with the native form. It is, however, not difficult to set up an effective standard screen for modified ghrelin molecules which fulfil the minimum requirements for immunological reactivity discussed herein. Hence, another part of the invention concerns a method for the identification of a modified ghrelin polypeptide which is capable of inducing antibodies against unmodified ghrelin in an animal species where the unmodified ghrelin polypeptide is a self-protein, the method comprising preparing, by means of peptide synthesis or by molecular biological means, a set of mutually distinct modified ghrelin polypeptides wherein amino acids have been added to, inserted in, deleted from, or substituted into the amino acid sequence of a ghrelin polypeptide of the animal species thereby giving rise to amino acid sequences in the set which comprise T-cell epitopes which are and foreign to the animal species, or preparing a set of nucleic acid fragments encoding the set of mutually distinct modified ghrelin polypeptides, testing members of the set for their ability to induce production of antibodies by the animal species against the unmodified ghrelin, and identifying and optionally isolating the member(s) of the set which significantly induces antibody production against unmodified ghrelin in the animal species, or identifying and optionally isolating the polypeptide expression products encoded by members of the set of nucleic acid fragments which significantly induces antibody production against unmodified ghrelin polypeptide in the animal species.

In this context, the "set of mutually distinct modified ghrelin polypeptides" is a collection of non-identical modified ghrelin polypeptides which have e.g. been selected on the basis of the criteria discussed above (e.g. in combination with studies of circular dichroism, NMR spectra, and/or ghrelin-ray diffraction patterns). The set may consist of only a few members but it is contemplated that the set may contain several hundred members. Likewise, the set of nucleic acid fragments is a collection of non-identical nucleic acid fragments, each encoding a modified ghrelin polypeptide selected in the same manner.

The test of members of the set can ultimately be performed *in vivo*, but a number of *in vitro* tests can be applied which narrow down the number of modified molecules which will serve the purpose of the invention.

Since the goal of introducing the foreign T-cell epitopes is to support the B-cell response by T-cell help, a prerequisite is that T-cell proliferation is induced by the modified ghrelin. T-cell proliferation can be tested by standardized proliferation assays *in vitro*. In short, a sample enriched for T-cells is obtained from a subject and subsequently kept in culture. The cultured T-cells are contacted with APCs of the subject which have previously taken up the modified molecule and processed it to present its T-cell epitopes. The proliferation of T-cells is moni-

tored and compared to a suitable control (e.g. T-cells in culture contacted with APCs which have processed intact, native ghrelin). Alternatively, proliferation can be measured by determining the concentration of relevant cytokines released by the T-cells in response to their recognition of foreign T-cells.

- 5 Having rendered highly probable that at least one modified ghrelin of the set is capable of inducing antibody production against ghrelin, it is possible to prepare an immunogenic composition comprising at least one modified ghrelin polypeptide which is capable of inducing antibodies against unmodified ghrelin in an animal species where the unmodified ghrelin polypeptide is a self-protein, the method comprising admixing the member(s) of the set which
- 10 significantly induces production of antibodies in the animal species which are reactive with ghrelin with a pharmaceutically and immunologically acceptable carrier and/or vehicle and/or diluent and/or excipient, optionally in combination with at least one pharmaceutically and immunologically acceptable adjuvant.

- Likewise, it is also possible to prepare an immunogenic composition which as an immunogen
- 15 contains a nucleic acid fragment encoding an immunogenic ghrelin analogue, cf. the discussion of nucleic acid vaccination above.

- The above aspects of the invention are conveniently carried out by initially preparing a number of mutually distinct nucleic acid sequences or vectors of the invention, inserting these into appropriate expression vectors, transforming suitable host cells with the vectors, and expressing the nucleic acid sequences of the invention. These steps can be followed by isolation of
- 20 the expression products. It is preferred that the nucleic acid sequences and/or vectors are prepared by methods comprising exercise of a molecular amplification technique such as PCR or by means of nucleic acid synthesis.

Coupling ghrelin to a polyhydroxypolymer

- 25 Molecules comprising a T helper epitope and peptides representing or including B-cell epitopes linked covalently to a non-immunogenic polymer molecule acting as a vehicle, e.g. a multivalent activated poly-hydroxypolymer, will function as a vaccine molecule that only contains the immunologically relevant parts, can be obtained. Promiscuous or so-called universal T-helper epitopes can be used if e.g. the target for the vaccine is a self-antigen. Furthermore, elements that enhance the immunological response could be also co-coupled to the vehicle and
- 30 thereby act as an adjuvant. Such elements could be mannose, tuftsin, muramyl dipeptide, CpG motifs etc, e.g. immune stimulating or targeting peptides. In that case, subsequent adjuvant formulation of the vaccine product might be unnecessary and the product could be administered in pure water or saline.

By coupling cytotoxic T cell (CTL) epitopes together with the T-helper epitopes it will also be possible to generate CTL's specific for the antigen from which the CTL epitope was derived. Elements that promote uptake of the product to the cytosol, such as mannose, of the APC, e.g. a macrophage, could also be co-coupled to the vehicle together with the CTL- and the T helper epitope and enhance the CTL response.

The ratio of B-cell epitopes and T-helper epitopes (P2 and P30) in the final product can be varied by varying the concentration of these peptides in the synthesis step. As mentioned above, the immunogenic molecule can be tagged with e.g. mannose, tuftsin, CpG-motifs or other immune stimulating substances (described herein) by adding these, if necessary by using e.g. aminated derivatives of the substances, to the carbonate buffer in the synthesis step.

If an insoluble activated polyhydroxy polymer is used to combine the peptides containing the B-cell epitope and the T-helper epitopes it can, as mentioned above be performed as a solid phase synthesis and the final product can be harvested and purified by wash and filtration. The elements to be coupled to a tresyl activated polyhydroxypolymer (peptides, tags etc) can be added to the polyhydroxypolymer at low pH, e.g. pH 4-5, and allowed to be equally distributed in the "gel" by passive diffusion. Subsequently, the pH can be raised to pH 9-10 to start the reaction of the primary amino groups on the peptides and tags to the tresyl groups on the polyhydroxy polymer. After coupling of peptides and e.g. immune stimulating elements the gel is grinded to form particles of suitable size for immunization.

This particular part of the invention therefore generally relates to an immunogen that comprises at least one first amino acid sequence derived from a protein of interest, wherein the at least one first amino acid sequence contains at least one B-cell and/or at least one CTL epitope, and at least one second amino acid sequence that includes a foreign T helper cell epitope, wherein each of the at least first and at least second amino acid sequences are coupled to a pharmaceutically acceptable activated polyhydroxypolymer carrier.

In order for the amino acid sequences to couple to the polyhydroxypolymer it is normally necessary to "activate" the polyhydroxypolymer with a suitable reactive group that can form the necessary link to the amino acid sequences.

The term "polyhydroxypolymer" is intended to have the same meaning as in WO 00/05316, i.e. the polyhydroxypolymer can have exactly the same characteristics as is specifically taught in that application. Hence, the polyhydroxypolymer can be water soluble or insoluble (thus requiring different synthesis steps during preparation of the immunogen). The polyhydroxypolymer can be selected from naturally occurring polyhydroxy compounds and synthetic polyhydroxy compounds.

Specific and preferred polyhydroxypolymers are polysaccharides selected from acetan, amylopectin, gum agar-agar, agarose, alginates, gum Arabic, carageenan, cellulose, cyclodextrins, dextran, furcellaran, galactomannan, gelatin, ghatti, glucan, glycogen, guar, karaya, konjac/A, locust bean gum, mannan, pectin, psyllium, pullulan, starch, tamarine, tragacanth, xanthan, xylan, and xyloglucan. Dextran is especially preferred.

However, the polyhydroxypolymer can also be selected from highly branched poly(ethyleneimine)(PEI), tetrathienylene vinylene, Kevlar (long chains of poly-paraphenyl terephthalamide), Poly(urethanes), Poly(siloxanes), polydimethylsiloxane, silicone, Poly(methyl methacrylate) (PMMA), Poly(vinyl alcohol), Poly(vinyl pyrrolidone), Poly(2-hydroxy ethyl methacrylate), Poly(N-vinyl pyrrolidone), Poly(vinyl alcohol), Poly(acrylic acid), Polytetrafluoroethylene (PTFE), Polyacrylamide, Poly(ethylene-co-vinyl acetate), Poly(ethylene glycol) and derivatives, Poly(methacrylic acid), Polylactides (PLA), Polyglycolides (PGA), Poly(lactide-co-glycolides) (PLGA), Polyanhydrides, and Polyorthoesters.

The (weight) average molecular weight of the polyhydroxypolymer in question (i.e. before activation) is typically at least 500, such as at least 1,000, preferably in the range of 2,500-2,000,000, more preferably in the range of 3,000-1,000,000, in particular in the range of 5,000-500,000. It has been shown in the examples that polyhydroxypolymers having an average molecular weight in the range of 10,000-200,000 are particularly advantageous.

The polyhydroxypolymer is preferably water soluble to an extent of at least 10 mg/ml, preferably at least 25 mg/ml, such as at least 50 mg/ml, in particular at least 100 mg/ml, such as at least 150 mg/ml at room temperature. It is known that dextran, even when activated as described herein, fulfils the requirements with respect to water solubility.

For some of the most interesting polyhydroxypolymers, the ratio between C (carbon atoms) and OH groups (hydroxy groups) of the unactivated polyhydroxypolymers (i.e. the native polyhydroxypolymer before activation) is in the range of 1.3 to 2.5, such as 1.5-2.3, preferably 1.6-2.1, in particular 1.85-2.05. Without being bound to any specific theory, it is believed that such a C/OH ratio of the unactivated polyhydroxypolymer represents a highly advantageous level of hydrophilicity. Polyvinylalcohol and polysaccharides are examples of polyhydroxypolymers which fulfil this requirement. It is believed that the above-mentioned ratio should be roughly the same for the activated polyhydroxypolymer as the activation ratio should be rather low.

The term "polyhydroxypolymer carrier" is intended to denote the part of the immunogen that carries the amino acid sequences. As a general rule, the polyhydroxypolymer carrier has its outer limits where the amino acid sequences can be cleaved off by a peptidase, e.g. in an antigen presenting cell that is processing the immunogen. Hence, the polyhydroxypolymer car-

rier can be the polyhydroxypolymer with an activation group, where the bond between the activation group and the amino acid sequence is cleavable by a peptidase in an APC, or the polyhydroxypolymer carrier can be a polyhydroxypolymer with activation group and e.g. a linker such as a single L-amino acid or a number of D-amino acids, where the last part of the
 5 linker can bond to the amino acid sequences and be cleaved by a peptidase in an APC.

As mentioned above, the polyhydroxypolymers carry functional groups (activation groups), which facilitates the anchoring of peptides to the carrier. A wide range of applicable functional groups are known in the art, e.g. tresyl (trifluoroethylsulphonyl), maleimido, p-nitrophenyl chloroformate, cyanogenbromide, tosyl (p-toluenesulfonyl), triflyl (trifluoromethanesulfonyl),
 10 pentafluorobenzenesulfonyl, and vinyl sulphone groups. Preferred examples of functional groups within the present invention are tresyl, maleimido, tosyl, triflyl, pentafluorobenzene-sulfonyl, p-nitrophenyl chloroformate, and vinylsulphone groups, among which tresyl, maleimido, and tosyl groups are particularly relevant.

Tresyl activated polyhydroxypolymers can be prepared using tresyl chloride as described for
 15 activation of dextran in Example 1 in WO 00/05316 or as described in Gregorius *et al.*, J. Immunol. Meth. 181 (1995) 65-73.

Maleimido activated polyhydroxypolymers can be prepared using *p*-maleimidophenyl isocyanate as described for activation of dextran in Example 3 of WO 00/05316. Alternatively, maleimido groups could be introduced to a polyhydroxypolymer, such as dextran, by derivati-
 20 sation of a tresyl activated polyhydroxypolymer (such as tresyl activated dextran (TAD)) with a diamine compound (generally $H_2N-C_nH_{2n}-NH_2$, where *n* is 1-20, preferably 1-8), e.g. 1,3-diaminopropane, in excess and subsequently react the amino groups introduced in TAD with reagents such as succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), sulfo-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), succinimi-
 25 dyl 4-(*p*-maleimidophenyl)butyrate (SMPB), sulfo-succinimidyl 4-(*p*-maleimidophenyl)butyrate (sulfo-SMPB), *N*- γ -maleimido butyryloxy-succinimide ester (GMBS) or *N*- γ -maleimido butyryl-oxy-sulfosuccinimide ester. Although the different reagents and routes for activation formally results in slightly different maleimide activated products with respect to the linkage between the maleimide functionality and the remainder of the parent hydroxy group on which activa-
 30 tion is performed, all and every are considered as "maleimide activated polyhydroxypolymers".

Tosyl activated polyhydroxypolymers can be prepared using tosyl chloride as described for activation of dextran in Example 2 in WO 00/05316. Triflyl and pentafluorobenzenesulfonyl activated polyhydroxypolymers are prepared as the tosyl or tresyl activated analogues, e.g.
 35 by using the corresponding acid chlorides.

Cyanogenbromide activated polyhydroxypolymer can be prepared by reacting the polyhydroxypolymer with cyanogenbromide using conventional methods. The resulting functional groups are normally cyanate esters with two hydroxy groups of the polyhydroxypolymer.

The degree of activation can be expressed as the ratio between the free hydroxy groups and the activation groups (i.e. functionalised hydroxy groups). It is believed that a ratio between the free hydroxy groups of the polyhydroxypolymer and the activation groups should be between 250:1 and 4:1 in order to obtain an advantageous balance between the hydrophilicity and the reactivity of the polyhydroxypolymer. Preferably the ratio is between 100:1 and 6:1, more preferably between 60:1 and 8:1, in particular between 40:1 and 10:1.

Especially interesting activated polyhydroxypolymers for use in the method for producing the generally applicable immunogen according to the invention are tresyl, tosyl and maleimido activated polysaccharides, especially tresyl activated dextran (TAD), tosyl activated dextran (TosAD), and maleimido activated dextran (MAD).

It is preferred that the bond between the polyhydroxypolymer carrier and the amino acid sequences attached thereto are cleavable by a peptidase, e.g. as a peptidase active in the processing of antigens in an APC. It is therefore preferred that the at least first and at least second amino acid sequences are coupled to the activated polyhydroxypolymer carrier via an amide bond or a peptide bond. It is especially preferred that the at least first and at least second amino acid sequences each provide for the nitrogen moiety of their respective amide bond.

The polyhydroxypolymer carrier may be substantially free of amino acid residues, necessitating that the activation group provides for part of a peptidase cleavable bond, but as mentioned above, the carrier may also simply include a spacer including at least one L-amino acid. Nevertheless, the at least first and at least second amino acid sequences are normally bound to the activated version of the polyhydroxypolymer via the nitrogen at the N-terminus of the amino acid sequence.

EXAMPLE 1

Immunization of rats with immunogenized rat ghrelin

5 peptides were produced by standard methods on a peptide synthesis apparatus. The peptides were: peptide 1, an irrelevant control peptide derived from IgE and also including the P2 and P30 epitopes (SEQ ID NO: 7 and 8, respectively), peptide 2, mature rat ghrelin, SEQ ID NO: 9, residues 24-53, and peptides 3-5, i.e. SEQ ID NOs: 15-17, respectively.

The 5 peptides were injected in 5 groups (n=10) of male Sprague-Dawley rats. Four injections were given subcutaneously with 3 week intervals, containing 100 µg of peptide in a total volume of 400 µl (Incl. adjuvant; complete Freund's Adjuvant, CFA, in first immunization, incomplete Freund's Adjuvant, IFA, in the booster immunizations).

- 5 Blood samples were drawn following 18 hours of fasting at the study initiation and following one week after each injection.

Blood samples were analysed for plasma Rat-Ghrelin using a commercial RIA kit from Phoenix (Ghrelin (Rat Mouse) - RIA Kit) according to the manufacturer's instructions, cf. Fig. 4. Anti-Ghrelin titres were measured using an ELISA where the binding of anti-Ghrelin antibodies to
10 wt Rat Ghrelin (Bachem) was detected with an HRP-conjugated anti-rat immunoglobulin secondary antibody (Dako), cf. Fig. 1.

Body weight as well as food and water intake was measured once a week, cf. Figs 2 and 3, respectively.

The results show that peptides 3-5 are capable of inducing antibodies reactive with rat
15 ghrelin, whereas the irrelevant control (peptide 1) and the wildtype ghrelin (peptide 2) are not. However, surprisingly, the antibody induction is correlated with increases in ghrelin levels in sera of the immunized rats and with an increase in body weight, whereas the food intake in the 5 groups of rats did not differ significantly.

The increase in body weight can therefore not be ascribed to an increase in food-intake but
20 must probably be ascribed to a change in the metabolism in the animals demonstrating antibodies against ghrelin.

EXAMPLE 2

Vaccine pilot studies with further variants

The study in Example 1 can in principal be made with several other candidate vaccine
25 molecules. The exemplary candidates for a ghrelin autovaccine can e.g. be constructed according to the above described concepts (described i.a. in detail in WO 95/05849) by substitution or insertion with known promiscuous T cell epitopes into the ghrelin wild type protein (or into its propeptide variant). The substitutions are peptide substitutions, where the inserted peptide may be of the same or different length than the deleted peptide in the wild-
30 type sequence.

For initial proof of concept by *in vivo* testing and screening, the constructs set forth in SEQ ID NOs: 1-5 may be used in mice. Corresponding variants can be made with the P2 and P30 epitopes substituting the PADRE sequence.

These constructs will be prepared synthetically by means of solid-phase peptide synthesis.

- 5 This will *i.e.* ensure that the constructs will lack mature ghrelin's biological activity, since it appears that the n-octanoylation of serine-3 (in mature ghrelin) is essential for ghrelin's biological activity. Of course, this effect can also be attained by utilising a recombinant expression system that does not allow for this particular post-translational modification.

- 10 A population of experimental animals will be vaccinated according to a standard protocol (priming with construct formulated in complete Freund's adjuvant and boosting with construct formulated in incomplete Freund's adjuvant) such as the one used in Example 1 with optimised amounts of these 5 constructs formulated according to standard procedures and animals will be compared to a control group with respect to weight gain/loss over time. Variants that produce a loss in weight (and also a decrease in ghrelin levels) will be suitable as
- 15 candidates for indications where weight loss is of interest, whereas the variants that mimic the results in Example 1 will have the other herein described applications.

CLAIMS

1. A method for immunizing against autologous ghrelin in an animal, including a human being, the method comprising effecting presentation to the animal's immune system of an immunogenically effective amount of an immunogen selected from the group consisting of
 - 5 - at least one ghrelin polypeptide or subsequence thereof which has been formulated so that immunization of the animal with the ghrelin polypeptide or subsequence thereof induces production of antibodies against the animal's autologous ghrelin, and
 - at least one ghrelin analogue that incorporates into the same molecule at least one B-cell epitope of ghrelin and at least one chemical moiety not derived from ghrelin so that immunization of the animal with the analogue induces production of antibodies against ghrelin.
- 10 2. The method according to claim 1, wherein the immunogen is a ghrelin analogue.
3. The method according to claim 2, wherein the analogue has preserved a substantial fraction of ghrelin B-cell epitopes and wherein the analogue also comprises
 - at least one foreign T helper lymphocyte epitope (T_H epitope), and/or
- 15 - at least one first moiety which effects targeting of the analogue to an antigen presenting cell (APC) or a B-lymphocyte, and/or
 - at least one second moiety which stimulates the immune system, and/or
 - at least one third moiety which optimises presentation of the analogue to the immune system.
- 20 4. The method according to claim 3, wherein the foreign T_H epitope and/or the first and/or the second and/or the third moiety is/are present in the analogue by being bound to suitable side groups ghrelin or a subsequence thereof.
5. The method according to claim 3 or 4, wherein the analogue is a ghrelin polypeptide that is modified by at least one amino acid substitution and/or deletion and/or insertion and/or addition.
- 25 6. The method according to claim 5, wherein the analogue is a fusion polypeptide.

7. The method according to claim 5 or 6, wherein the amino acid substitution and/or deletion and/or insertion and/or addition allows for a substantial preservation of the overall tertiary structure of ghrelin in the analogue.
8. The method according to any one of claims 2-7, wherein the analogue includes duplication
5 of at least one ghrelin B-cell epitope and/or introduction of a hapten.
9. The method according to any one of claims 3-8, wherein the foreign T-cell epitope is immunodominant in the animal.
10. The method according to any one of claims 3-9, wherein the foreign T-cell epitope is promiscuous.
- 10 11. The method according to claim 10, wherein the at least one foreign T-cell epitope is selected from a natural promiscuous T-cell epitope and an artificial MHC-II binding peptide sequence.
12. The method according to claim 11, wherein the natural T-cell epitope is selected from a Tetanus toxoid epitope such as P2 or P30, a diphtheria toxoid epitope, an influenza virus hemagglutinin epitope, and a *P. falciparum* CS epitope.
15
13. The method according to any one of claims 3-12, wherein the first moiety is a substantially specific binding partner for a B-lymphocyte specific surface antigen or for an APC specific surface antigen, such as a hapten or a carbohydrate for which there is a receptor on the B-lymphocyte or the APC.
- 20 14. The method according to any one of claims 3-13, wherein the second moiety is selected from a cytokine and a heat-shock protein.
15. The method according to claim 6, wherein the cytokine is selected from, or is an effective part of, Interferon γ (IFN- γ), Flt3L, Interleukin 1 (IL-1), Interleukin 2 (IL-2), Interleukin 4 (IL-4), Interleukin 6 (IL-6), Interleukin 12 (IL-12), Interleukin 13 (IL-13), Interleukin 15 (IL-15),
25 and granulocyte-macrophage colony stimulating factor (GM-CSF), and the heat-shock protein is selected from, or is an effective part of any of, HSP70, HSP90, HSC70, GRP94, and calreticulin (CRT).
16. The method according to any one of claims 3-15, wherein the third moiety is of lipid nature, such as a palmitoyl group, a myristyl group, a farnesyl group, a geranyl-geranyl group,
30 a GPI-anchor, and an N-acyl diglyceride group.

17. The method according to any of the preceding claims wherein the immunogen comprises a substitution of at least one amino acid sequence within the ghrelin polypeptide with an amino acid sequence of equal or different length which gives rise to a foreign T_H epitope in the analogue.
- 5 18. The method according to any of the preceding claims, wherein the ghrelin polypeptide comprises an amino acid sequence corresponding to amino acids 24-51 in SEQ ID NO: 11 or a subsequence thereof, wherein is inserted an amino acid sequence that gives rise to a foreign T_H epitope in the analogue or wherein at least one amino acid sequence is substituted by an amino acid sequence of equal or different length so as to give rise to a foreign T_H epitope in
10 the analogue, wherein the introduction is performed after any one of amino acids 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100,
15 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, and 117 in SEQ ID NO: 11, and wherein amino acid 1 and/or 2 and/or 3 and/or 4 and/or 5 and/or 6 and/or 7 and/or 8 and/or 9 and/or 10 and/or 11 and/or 12 and/or 13 and/or 14 and/or 15 and/or 16 and/or 17 and/or 18 and/or 19 and/or 20 and/or 21 and/or 22 and/or 23 and/or 24 and/or 25 and/or 26 and/or 27 and/or 28 and/or 29 and/or 30 and/or 31 and/or 32 and/or 33
20 and/or 34 and/or 35 and/or 36 and/or 37 and/or 38 and/or 39 and/or 40 and/or 41 and/or 42 and/or 43 and/or 44 and/or 45 and/or 46 and/or 47 and/or 48 and/or 49 and/or 50 and/or 51 and/or 52 and/or 53 and/or 54 and/or 55 and/or 56 and/or 57 and/or 58 and/or 59 and/or 60 and/or 61 and/or 62 and/or 63 and/or 64 and/or 65 and/or 66 and/or 67 and/or 68 and/or 69 and/or 70 and/or 71 and/or 72 and/or 73 and/or 74 and/or 75 and/or 76 and/or 77 and/or 78
25 and/or 79 and/or 80 and/or 81 and/or 82 and/or 83 and/or 84 and/or 85 and/or 86 and/or 87 and/or 88 and/or 89 and/or 90 and/or 91 and/or 92 and/or 93 and/or 94 and/or 95 and/or 96 and/or 97 and/or 98 and/or 99 and/or 100 and/or 101 and/or 102 and/or 103 and/or 104 and/or 105 and/or 106 and/or 107 and/or 108 and/or 109 and/or 110 and/or 111 and/or 112 and/or 113 and/or 114 and/or 115 and/or 116 and/or 117 in SEQ ID NO: 11 may be deleted.
- 30 19. The method according to claim 23, wherein the analogue is selected from the group consisting of polypeptides having an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5.
20. The method according to claim 20, wherein the immunogen has polyamino acids covalently or non-covalently linked to a carrier molecule capable of effecting presentation of multiple
35 copies of antigenic determinants, wherein the polyamino acids are selected from the group consisting of a ghrelin polypeptide, a ghrelin subsequence, and a ghrelin analogue.

21. The method according to claim 20, wherein the carrier molecule contains or consists of a pharmaceutically acceptable activated polyhydroxypolymer.
22. The method according to claim 21 insofar as it depends on claim 3, wherein the polyhydroxypolymer serves as a carrier backbone to which are separately bound 1) a ghrelin polypeptide or subsequence thereof and 2) a foreign T_H epitope.
23. The method according to claim 21 or 22, wherein the polyamino acids are bound to the polyhydroxypolymer via a bond cleavable by a peptidase, such as an amide bond or a peptide bond.
24. The method according to claim 23, wherein the polyamino acids provide for the nitrogen moiety of their respective amide bond.
25. The method according to any one of claims 21-24, wherein the polyhydroxypolymer carrier is substantially free of amino acid residues.
26. The method according to any one of claims 21-25, wherein the polyamino acids are bound to the activated polyhydroxypolymer via the nitrogen at the N-terminus of the amino acid sequence.
27. The method according to any one of claims 21-26 wherein the polyhydroxypolymer is water soluble.
28. The method according to any one of claims 21-26 wherein the polyhydroxypolymer is water insoluble.
29. The method according to any one of claims 21-28, wherein the polyhydroxypolymer is selected from naturally occurring polyhydroxy compounds and synthetic polyhydroxy compounds.
30. The method according to any one of claims 21-29, wherein the polyhydroxypolymer is a polysaccharide.
31. The method according to claim 30, wherein the polysaccharide is selected from the group consisting of acetan, amylopectin, gum agar-agar, agarose, alginates, gum Arabic, carrageenan, cellulose, cyclodextrins, dextran, furcellaran, galactomannan, gelatin, ghatti, glucan, glycogen, guar, karaya, konjac/A, locust bean gum, mannan, pectin, psyllium, pullulan, starch, tamarine, tragacanth, xanthan, xylan, and xyloglucan.

32. The method according to claim 31, wherein the polyhydroxypolymer is dextran.

33. The method according to any one of claims 21-29, wherein the polyhydroxypolymer is selected from the group consisting of highly branched poly(ethylenimine)(PEI), tetrathienylene vinylene, Kevlar (long chains of poly-paraphenyl terephthalamide), Poly(urethanes),

5 Poly(siloxanes), polydimethylsiloxane, silicone, Poly(methyl methacrylate) (PMMA), Poly(vinyl alcohol), Poly(vinyl pyrrolidone), Poly(2-hydroxy ethyl methacrylate), Poly(N-vinyl pyrrolidone), Poly(vinyl alcohol), Poly(acrylic acid), Polytetrafluoroethylene (PTFE), Polyacrylamide, Poly(ethylene-co-vinyl acetate), Poly(ethylene glycol) and derivatives, Poly(methacrylic acid), Poly(lactides (PLA), Polyglycolides (PGA), Poly(lactide-co-glycolides) (PLGA), Polyanhydrides,
10 and Polyorthoesters.

34. The method according to any of claims 21-33, wherein the average molecular weight of the polyhydroxypolymer before activation is at least 500.

35. The method according to any one of claims 21-34, wherein the polyhydroxypolymer is activated with functional groups selected from tresyl (trifluoroethylsulphonyl), maleimido, p-
15 nitrophenyl chloroformate, and tosyl (p-toluenesulfonyl).

36. The method according to any of claims 21-35 that further comprises at least one further polyamino acid is coupled to the polyhydroxypolymer, said at least one further polyamino acid being selected from the group consisting of an immune stimulating peptide or a targeting peptide.

20 37. The method according to any one of the preceding claims, wherein an effective amount of the immunogen is administered to the animal via a route selected from the parenteral route such as the intradermal, the subdermal, the intracutaneous, the subcutaneous, and the intramuscular routes; the peritoneal route; the oral route; the buccal route; the sublingual route; the epidural route; the spinal route; the anal route; and the intracranial route.

25 38. The method according to claim 28, wherein the effective amount is between 0.5 µg and 2,000 µg of the ghrelin polypeptide, the subsequence thereof or the analogue thereof.

39. The method according to claim 28 or 29, wherein the ghrelin polypeptide or analogue is contained in a virtual lymph node (VLN) device.

30 40. The method according to any one of claims 20-39, wherein the ghrelin polypeptide, the subsequence thereof, or the ghrelin analogue has been formulated with an adjuvant which facilitates breaking of autotolerance to autoantigens.

41. The method according to any one of claims 1-19, wherein presentation of the immunogen to the Immune system is effected by introducing nucleic acid(s) encoding the immunogen into the animal's cells and thereby obtaining *in vivo* expression by the cells of the nucleic acid(s) introduced.
- 5 42. The method according to claim 41, wherein the nucleic acid(s) introduced is/are selected from naked DNA, DNA formulated with charged or uncharged lipids, DNA formulated in liposomes, DNA included in a viral vector, DNA formulated with a transfection-facilitating protein or polypeptide, DNA formulated with a targeting protein or polypeptide, DNA formulated with Calcium precipitating agents, DNA coupled to an inert carrier molecule, DNA encapsulated in
10 chitin or chitosan, and DNA formulated with an adjuvant.
43. The method according to claim 42, wherein the nucleic acid(s) is/are contained in a VLN device.
44. The method according to any one of claims 37-43, which includes at least one administration/introduction per year, such as at least 2, at least 3, at least 4, at least 6, and at least 12
15 administrations/introductions.
45. The method according to any one of the preceding claims, wherein autologous ghrelin is down-regulated as a consequence of the immunization.
46. The method according to any one of claims 1-44, wherein autologous ghrelin is up-regulated as a consequence of the immunization.
- 20 47. A method for treating and/or preventing and/or ameliorating obesity or other diseases and conditions characterized by excess body fat deposits, the method comprising down-regulating ghrelin according to the method of claim 45 to such an extent that the total amount of body fat is significantly decreased.
48. A method for increasing body mass in an animal, such as a human being, the method
25 comprising up-regulating autologous ghrelin in the animal according to the method of claim 46
49. An analogue of a ghrelin polypeptide which is derived from an animal ghrelin polypeptide wherein is introduced a modification which has as a result that immunization of the animal with the analogue induces production of antibodies against the animal's autologous ghrelin
30 polypeptide.
50. An analogue according to claim 49, which is as defined in any one of claims 3-36.

51. An immunogenic composition comprising

- an immunogenically effective amount of a ghrelin polypeptide autologous in an animal or a subsequence of said ghrelin polypeptide, said ghrelin polypeptide or subsequence thereof being formulated together with an immunologically acceptable adjuvant so as to break the animal's autotolerance towards the ghrelin polypeptide, the composition further comprising a pharmaceutically and immunologically acceptable carrier and/or vehicle, or
- an immunogenically effective amount of an analogue according to claim 49 or 50, the composition further comprising a pharmaceutically and immunologically acceptable carrier and/or vehicle and optionally an adjuvant.

10 52. A nucleic acid fragment which encodes an analogue as defined in any one of claims 3-19.

53. A vector carrying the nucleic acid fragment according to claim 52, such as a vector that is capable of autonomous replication.

54. The vector according to claim 53, which is selected from the group consisting of a plasmid, a phage, a cosmid, a mini-chromosome, and a virus.

15 55. The vector according to claim 53 or 54, comprising, in the 5'→3' direction and in operable linkage, a promoter for driving expression of the nucleic acid fragment according to claim 52, optionally a nucleic acid sequence encoding a leader peptide enabling secretion of or integration into the membrane of the polypeptide fragment, the nucleic acid fragment according to claim 51, and optionally a terminator.

20 56. The vector according to any one of claims 53-55 which, when introduced into a host cell, is capable or incapable of being integrated in the host cell genome.

57. The vector according to claim 55 or 56, wherein a promoter drives expression in a eukaryotic cell and/or in a prokaryotic cell.

25 58. A transformed cell carrying the vector of any one of claims 53-57, such as a transformed cell which is capable of replicating the nucleic acid fragment according to claim 52.

59. The transformed cell according to claim 60, which is a microorganism selected from a bacterium, a yeast, a protozoan, or a cell derived from a multicellular organism selected from a fungus, an insect cell such as an S₂ or an SF cell, a plant cell, and a mammalian cell.

60. The transformed cell according to claim 58 or 59, which expresses the nucleic acid fragment according to claim 52, such as a transformed cell, which secretes or carries on its surface, the analogue according to claim 49 or 50.

61. The method according to any one of claims 1-19, wherein presentation to the immune system is effected by administering a non-pathogenic microorganism or virus which is carrying a nucleic acid fragment which encodes and expresses the ghrelin polypeptide, subsequence or analogue.

62. A composition for inducing production of antibodies against a ghrelin polypeptide in the autologous host, the composition comprising

10 - a nucleic acid fragment according to claim 52 or a vector according to any one of claims 53-57, and

- a pharmaceutically and immunologically acceptable carrier and/or vehicle and/or adjuvant.

63. A stable cell line which carries the vector according to any one of claims 53-57 and which expresses the nucleic acid fragment according to claim 52, and which optionally secretes or carries the analogue according to claim 49 or 50 on its surface.

64. A method for the preparation of the cell according to any one of claims 58-60, the method comprising transforming a host cell with the nucleic acid fragment according to claim 52 or with the vector according to any one of claims 53-57.

65. A method for the identification of a modified ghrelin polypeptide which is capable of inducing antibodies against unmodified ghrelin polypeptide in an animal species where the unmodified ghrelin polypeptide is a self-protein, the method comprising

25 - preparing, by means of peptide synthesis or genetic engineering techniques, a set of mutually distinct modified ghrelin polypeptides wherein amino acids have been added to, inserted in, deleted from, or substituted into the amino acid sequence of a ghrelin polypeptide of the animal species thereby giving rise to amino acid sequences in the set which comprise T-cell epitopes which are foreign to the animal species, or preparing a set of nucleic acid fragments encoding the set of mutually distinct modified ghrelin polypeptides,

30 - testing members of the set of modified ghrelin polypeptides or nucleic acid fragments for their ability to induce production of antibodies by the animal species against the unmodified ghrelin polypeptide, and

- Identifying and optionally isolating the member(s) of the set of modified ghrelin polypeptides which significantly induces antibody production against unmodified ghrelin polypeptide in the species or identifying and optionally isolating the polypeptide expression products encoded by members of the set of nucleic acid fragments which significantly induces antibody production
5 against unmodified ghrelin polypeptide in the animal species.

66. A method for the preparation of an immunogenic composition comprising at least one modified ghrelin polypeptide which is capable of inducing antibodies against unmodified ghrelin polypeptide in an animal species where the unmodified ghrelin polypeptide is a self-protein, the method comprising

- 10 - preparing, by means of peptide synthesis or genetic engineering techniques, a set of mutually distinct modified ghrelin polypeptides wherein amino acids have been added to, inserted in, deleted from, or substituted into the amino acid sequence of a ghrelin polypeptide of the animal species thereby giving rise to amino acid sequences in the set comprising T-cell epitopes which are foreign to the animal,

- 15 - testing members of the set for their ability to induce production of antibodies by the animal species against the unmodified ghrelin polypeptide, and

- admixing the member(s) of the set which significantly induces production of antibodies in the animal species which are reactive with the ghrelin polypeptide with a pharmaceutically and immunologically acceptable carrier and/or vehicle, optionally in combination with at least

- 20 one pharmaceutically and immunologically acceptable adjuvant.

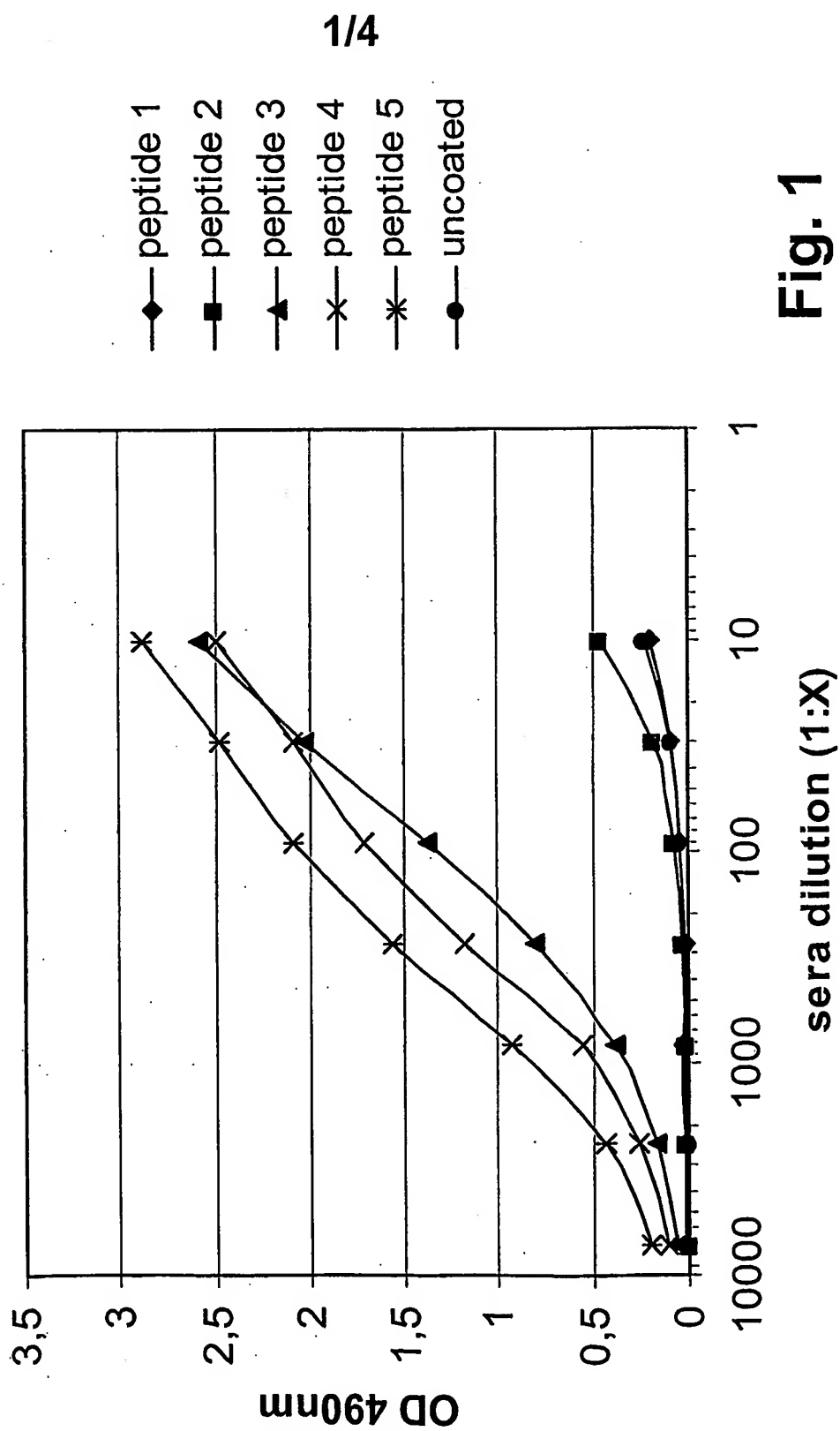
67. The method according to claim 65 or 66, wherein preparation of the members of the set comprises preparing mutually distinct nucleic acid sequences, each sequence being a nucleic acid sequence according to claim 62, inserting the nucleic acid sequences into appropriate expression vectors, transforming suitable host cells or host animals with the vectors, and effecting expression of the nucleic acid sequences, optionally followed by isolating the expression products.

68. The method according to claim 67, wherein the preparation of the nucleic acid sequences and/or the vectors is achieved by the aid of a molecular amplification technique such as PCR or by the aid of nucleic acid synthesis.

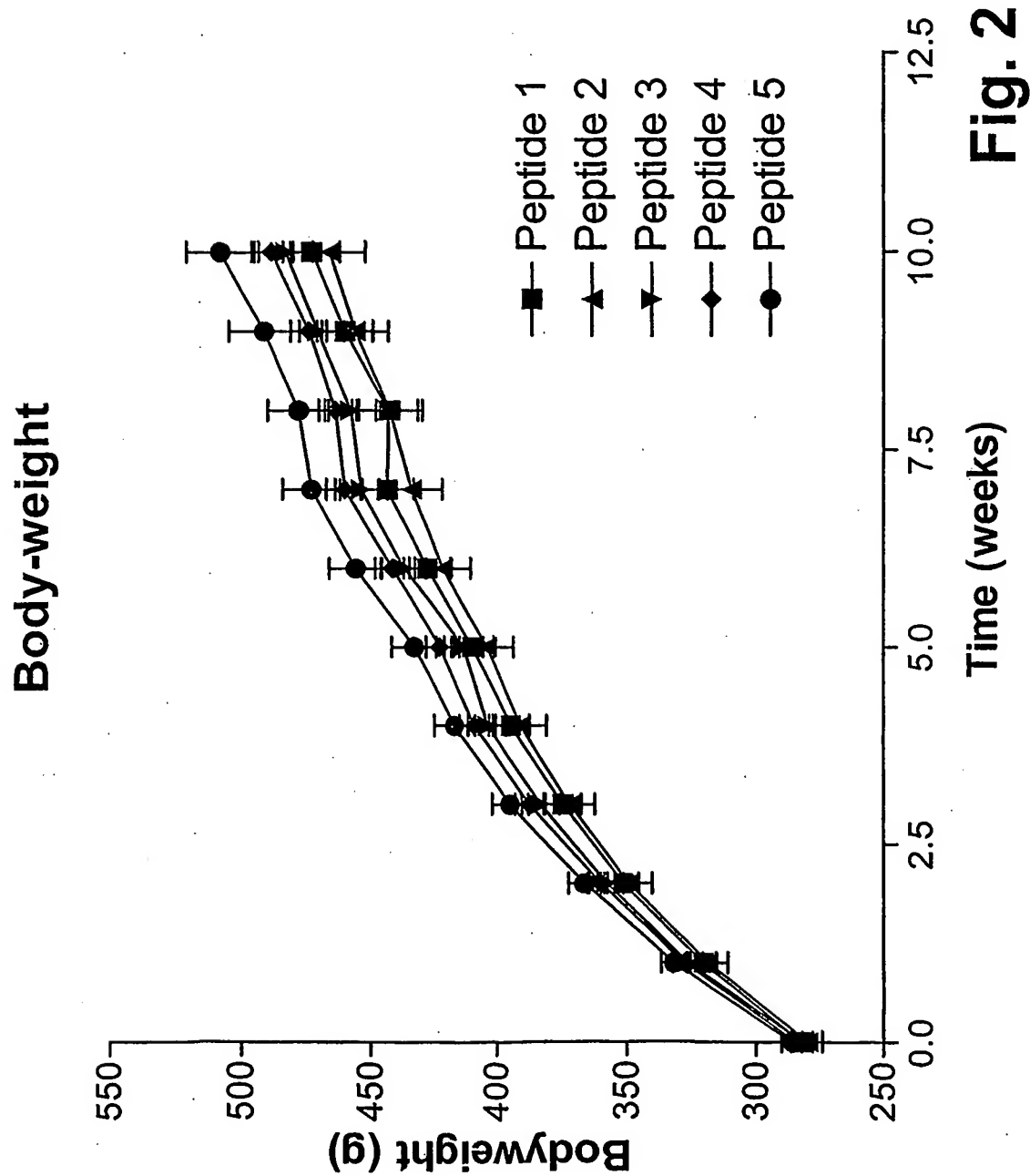
- 30 69. Use of a ghrelin polypeptide or a subsequence thereof for the preparation of an immunogenic composition comprising an adjuvant for down-regulating ghrelin in an animal.

70. Use of a ghrelin polypeptide or a subsequence thereof for the preparation of an immunogenic composition comprising an adjuvant for up-regulating ghrelin in an animal.
71. Use of a ghrelin polypeptide or a subsequence thereof for the preparation of an immunogenic composition comprising an adjuvant for the treatment, prophylaxis or amelioration of
5 obesity characterized by excess body fat deposits.
72. Use of a ghrelin polypeptide or a subsequence thereof for the preparation of an immunogenic composition comprising an adjuvant for treatment, prophylaxis or amelioration of anorexia, cachexia, wounds, or burns, or for adjuvating therapy to in vitro fertilization.
73. Use of an analogue of a ghrelin polypeptide for the preparation of an immunogenic
10 composition optionally comprising an adjuvant for down-regulating ghrelin in an animal.
74. Use of an analogue of a ghrelin polypeptide for the preparation of an immunogenic composition optionally comprising an adjuvant for the treatment, prophylaxis or amelioration of obesity characterized by excess body fat deposits.
75. Use of an analogue of a ghrelin polypeptide for the preparation of an immunogenic composition optionally comprising an adjuvant for up-regulating ghrelin in an animal.
15
76. Use of an analogue of a ghrelin polypeptide for the preparation of an immunogenic composition optionally comprising an adjuvant for treatment, prophylaxis or amelioration of anorexia, cachexia, wounds, or burns, or for adjuvating therapy to in vitro fertilization.

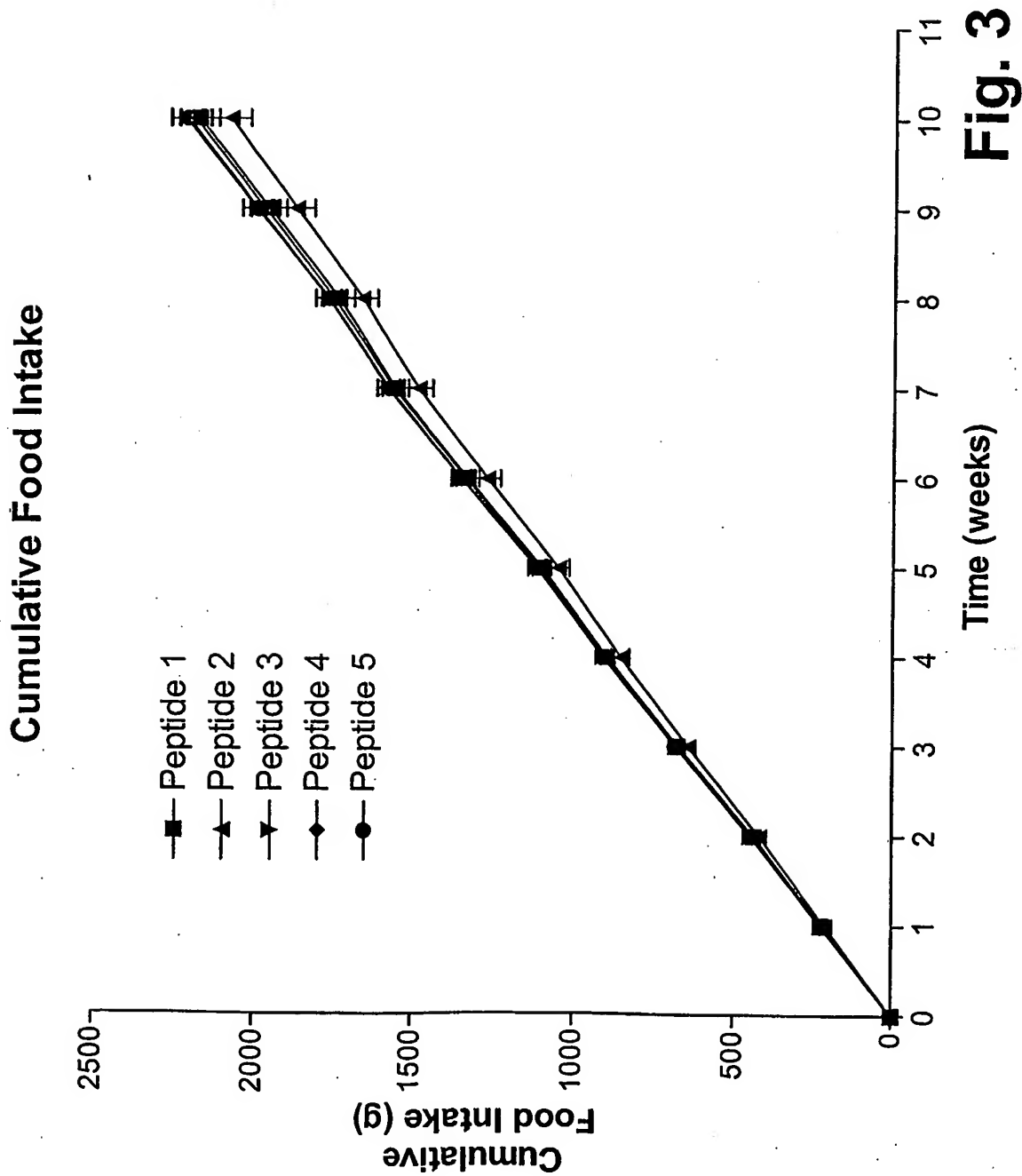
ELISA on anti Ghrelin antibody titers in sera pools



2/4

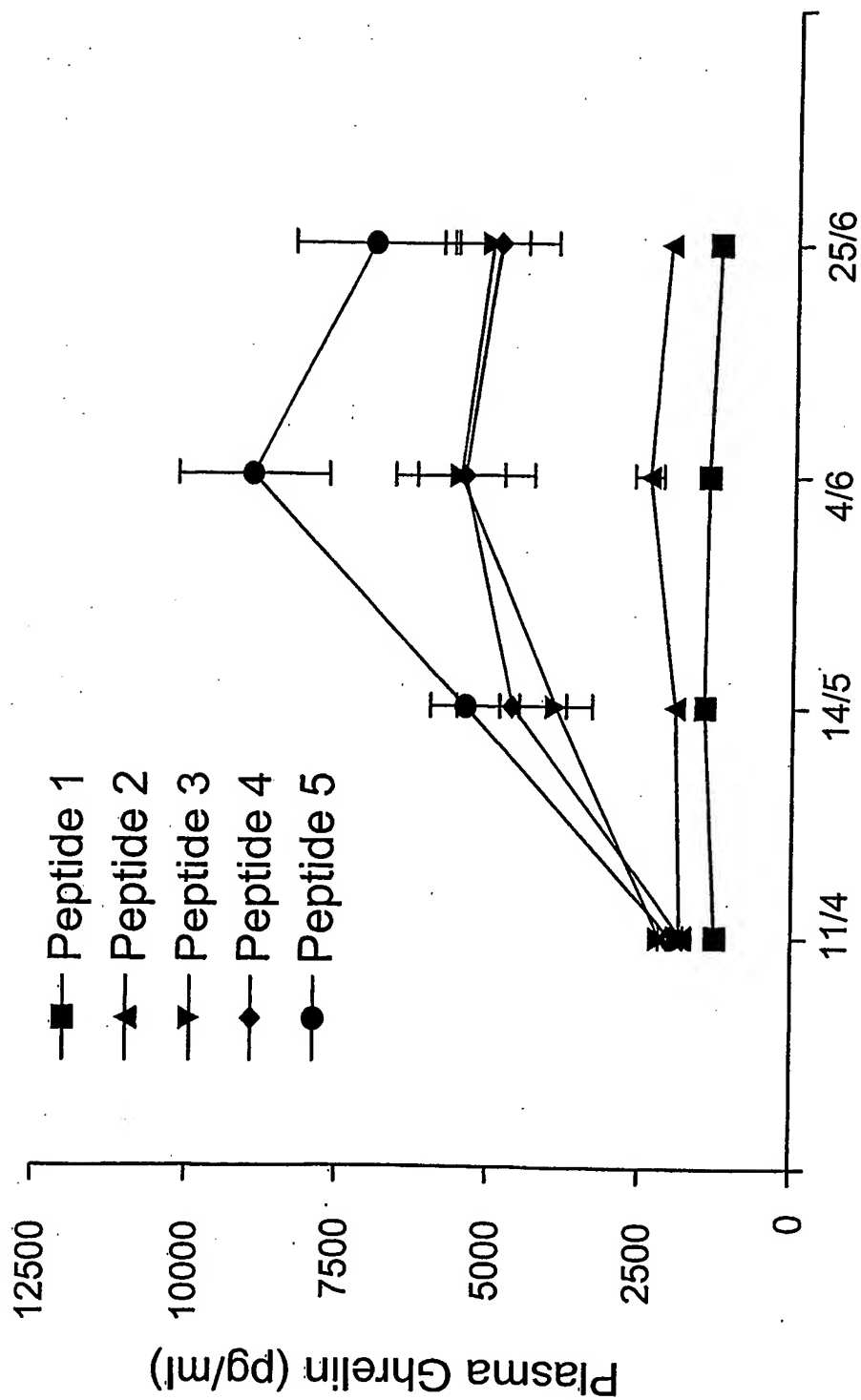
**Fig. 2**

3/4



4/4

Ghrelin levels in pooled rat sera

**Fig. 4**

Date of blood sampling

<212> PRT

<213> Artificial sequence

<220>

<223> Mature ghrelin with PADRE sequence inserted

<400> 3

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Lys Phe Val Ala
1 5 10 15

Ala Trp Thr Leu Lys Ala Ala Ala Ala Gln Gln Arg Lys Glu Ser Lys
20 25 30

Lys Pro Pro Ala Lys Leu Gln Pro Arg
35 40

<210> 4

<211> 41

<212> PRT

<213> Artificial sequence

<220>

<223> Mature ghrelin with PADRE sequence inserted

<400> 4

Gly Ser Ser Phe Leu Ser Ala Lys Phe Val Ala Ala Trp Thr Leu Lys
1 5 10 15

Ala Ala Ala Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys
20 25 30

Lys Pro Pro Ala Lys Leu Gln Pro Arg
35 40

<210> 5

<211> 40

<212> PRT

<213> Artificial sequence

<220>

<223> Mature ghrelin with PADRE sequence substituted in

<400> 5

Gly Ser Ser Phe Leu Ser Ala Lys Phe Val Ala Ala Trp Thr Leu Lys
1 5 10 15

Ala Ala Ala Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys Lys
20 25 30

Pro Pro Ala Lys Leu Gln Pro Arg
35 40

<210> 6
<211> 13
<212> PRT
<213> Artificial sequence

<220>
<223> Pan DR binding peptide, PADRE

<400> 6

Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
1 5 10

<210> 7
<211> 15
<212> PRT
<213> Clostridium tetani

<400> 7

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
1 5 10 15

<210> 8
<211> 21
<212> PRT
<213> Clostridium tetani

<400> 8

Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
1 5 10 15

Ala Ser His Leu Glu
20

<210> 9
<211> 501
<212> DNA
<213> Rattus rattus

<220>
<221> exon
<222> (31)..(384)
<223>

<220>
<221> misc_feature
<222> (31)..(381)
<223> Ghrelin pre-propeptide

```
<220>
<221> misc_feature
<222> (100)..(183)
<223> Mature ghrelin
```

```
<220>
<221> sig_peptide
<222> (31)..(99)
<223>
```

[illegible]

$\langle 210 \rangle$	10
$\langle 211 \rangle$	487
$\langle 212 \rangle$	DNA

<223>

<223> Ghrelin pre-propeptide

<223> Ghrelin propeptide

<223> Mature ghrelin

<223>

cag cat ggc cgg gcc ctg ggg aag ttt ctt cag gat atc ctc tgg gaa 339
Gln His Gly Arg Ala Leu Gly Lys Phe Leu Gln Asp Ile Leu Trp Glu
95 100 105

gag gtc aaa gag gcg cca gct gac aag taa ccacggacag gcctgacccc 389
 Glu Val Lys Glu Ala Pro Ala Asp Lys
 110 115

cgtgctttcc ttctcctgag caagaactca catccgcctc agcctcctcg gcaactocca 449

gcactctcct accactttta gaataaatgt tcacctgt 487

<210> 11
 <211> 511
 <212> DNA
 <213> Homo sapiens

<220>
 <221> exon
 <222> (34)..(387)
 <223>

<220>
 <221> misc_feature
 <222> (34)..(384)
 <223> Ghrelin pre-propeptide

<220>
 <221> misc_feature
 <222> (103)..(384)
 <223> Ghrelin propeptide

<220>
 <221> misc_feature
 <222> (103)..(186)
 <223> Mature ghrelin

<220>
 <221> sig_peptide
 <222> (34)..(102)
 <223>

<400> 11
 gcaggccac ctgtctgcaa cccagctgag gcc atg ccc tcc cca ggg acc gtc 54
 Met Pro Ser Pro Gly Thr Val
 1 5

tgc agc ctc ctg ctc ctc ggc atg ctc tgg ctg gac ttg gcc atg gca 102
 Cys Ser Leu Leu Leu Leu Gly Met Leu Trp Leu Asp Leu Ala Met Ala
 10 15 20

ggc tcc agc ttc ctg agc cct gaa cac cag aga gtc cag cag aga aag 150
 Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys
 25 30 35

gag tcg aag aag cca cca gcc aag ctg cag ccc cga gct cta gca ggc 198
 Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Ala Leu Ala Gly
 40 45 50 55

	1	5	
acc atg tgc agc ctg ctg ctc ttc agt gtg ctc tgg gtg gac ctg gcc			102
Thr Met Cys Ser Leu Leu Leu Phe Ser Val Leu Trp Val Asp Leu Ala			
	10	20	
atg gcg ggc tcc agc ttc cta agt ccc gaa cac cag aaa cta cag cag			150
Met Ala Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Gln			
	25	35	
aga aag gag tcc aag aag ccg ccg gcc aaa ctg cag ccc cga gcc cta			198
Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Ala Leu			
	40	50	
gaa ggc tcc ctt ggc cca gaa gac aca agt caa gtg gaa gag gca gag			246
Glu Gly Ser Leu Gly Pro Glu Asp Thr Ser Gln Val Glu Glu Ala Glu			
	55	65	
gat gag ctg gaa atc cgg ttc aat gcc ccc ttt gat gtt gga atc aag			294
Asp Glu Leu Glu Ile Arg Phe Asn Ala Pro Phe Asp Val Gly Ile Lys			
	70	80	85
ctg tca ggg cct cag tac cac cag cat ggc cag gca ctc ggg aag ttt			342
Leu Ser Gly Pro Gln Tyr His Gln His Gly Gln Ala Leu Gly Lys Phe			
	90	95	100
ctt caa gag gtt ctt tgg gaa gac acc aac gag gcc ctg gca gac gag			390
Leu Gln Glu Val Leu Trp Glu Asp Thr Asn Glu Ala Leu Ala Asp Glu			
	105	110	115
tga tcattccacaa gatgggcctg cctgtttctcc cccacccta gaagcactca			443
cctgactttt acactgtttc tgcagctact cccagttctg agtggtacta gttgaagagg			503
tgaataaaca ttcaaaccat aaaaaaaaaa aaaaaaaact cgag			547

<210> 13
 <211> 494
 <212> DNA
 <213> Sus scrofa

<220>
 <221> exon
 <222> (9)..(365)
 <223>

<220>
 <221> sig_peptide
 <222> (9)..(79)
 <223>

<220>
 <221> misc_feature
 <222> (9)..(362)
 <223> Ghrelin pre-propeptide

<220>

<221> misc_feature
 <222> (80)..(362)
 <223> Ghrelin propeptide

<220>
 <221> misc_feature
 <222> (80)..(164)
 <223> Mature ghrelin

<400> 13
 ctgaggcc atg ccc tcc acg ggg acc att tgc agc ctg ctg ctc ctc agc 50
 Met Pro Ser Thr Gly Thr Ile Cys Ser Leu Leu Leu Leu Ser
 1 5 10

gtg ctc ctc atg gca gac ttg gcc atg gcg ggc tcc agc ttc ttg agc 98
 Val Leu Leu Met Ala Asp Leu Ala Met Ala Gly Ser Ser Phe Leu Ser
 15 20 25 30

ccc gaa cac cag aaa gtg cag cag aga aag gag tcc aag aag cca gca 146
 Pro Glu His Gln Lys Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Ala
 35 40 45

gcc aaa ctg aag ccc cgg gcc ctg gaa ggc tgg ctc ggc cca gaa gac 194
 Ala Lys Leu Lys Pro Arg Ala Leu Glu Gly Trp Leu Gly Pro Glu Asp
 50 55 60

agt ggt gag gtg gaa ggc acg gag gac aag ctg gaa atc cgg ttc aac 242
 Ser Gly Glu Val Glu Gly Thr Glu Asp Lys Leu Glu Ile Arg Phe Asn
 65 70 75

gcc ccc tgt gat gtt ggg atc aag ttg tca ggg gct cag tcc gac cag 290
 Ala Pro Cys Asp Val Gly Ile Lys Leu Ser Gly Ala Gln Ser Asp Gln
 80 85 90

cac ggc cag ccc ctg ggg aaa ttt ctc cag gac atc ctc tgg gaa gag 338
 His Gly Gln Pro Leu Gly Lys Phe Leu Gln Asp Ile Leu Trp Glu Glu
 95 100 105 110

gtc act gag gcc ccg gcc gac aag tga ttgtccctga gaccagccac 385
 Val Thr Glu Ala Pro Ala Asp Lys
 115

ctctgttctc ccagcctcct aagggctcac ctggcttcca ggacgcttcc actatcacac 445
 ccagctctga gggatgctag cctgggaggt gaataaacat tcagactgg 494

<210> 14
 <211> 488
 <212> DNA
 <213> Bos taurus

<220>
 <221> exon
 <222> (4)..(354)
 <223>

```
<220>
<221>   sig_peptide
<222>   (4)..(72)
<223>
```

```
<220>
<221> misc_feature
<222> (4)..(351)
<223> Ghrelin pre-propeptide
```

```
<220>
<221> misc_feature
<222> (73)..(351)
<223> Ghrelin propeptide
```

```
<220>
<221> misc_feature
<222> (73)..(153)
<223> Mature ghrelin
```

[illegible]

<210> 15
 <211> 64
 <212> PRT
 <213> Artificial sequence

<220>
 <223> Mature rat ghrelin with added epitopes

<400> 15

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Gly
 1 5 10 15

Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys Glu
 20 25 30

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Phe Asn Asn Phe Thr
 35 40 45

Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu
 50 55 60

<210> 16
 <211> 68
 <212> PRT
 <213> Artificial sequence

<220>
 <223> Mature ghrelin with added epitopes

<400> 17

Glu Glu Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys
 1 5 10 15

Val Ser Ala Ser His Leu Glu Gly Ser Ser Phe Leu Ser Pro Glu His
 20 25 30

Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu
 35 40 45

Gln Pro Arg Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr
 50 55 60

Glu Leu Glu Glu
 65

<210> 17
 <211> 68

<212> PRT

<213> Artificial sequence

<220>

<223> Mature ghrelin with added epitopes

<400> 17

Glu Glu Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu
1 5 10 15

Leu Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg
20 25 30

Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Phe Asn Asn
35 40 45

Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His
50 55 60

Leu Glu Glu Glu
65

INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 03/00592

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/39 A61K39/385 A61K39/00 C07K14/435 A61P3/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 53312 A (CHEN RUI HONG ;GOODRICH RYLE (US); HYSEQ INC (US); WANG DUNRUI (US) 26 July 2001 (2001-07-26) page 39, line 1 - line 3 page 61, line 9 - line 11 page 74, line 16 - line 28 page 75, line 16 - line 31 page 76, line 14 - line 22 page 604; claims 1,6-8 page 149 SEQ ID NO. 249	1-64,69, 70,73,75
X	--- WO 02 36792 A (CATCHPOLE IAN RICHARD ;RHODES JOHN RICHARD (GB); GLAXO GROUP LTD () 10 May 2002 (2002-05-10) page 14, line 19 - line 36 page 16, line 14-17 --- -/-	1,2,49, 51,69, 70,73,75

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

Date of the actual completion of the international search

29 January 2004

Date of mailing of the international search report

17 02 2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Sara Nilsson

INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 03/00592

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2001/020012 A1 (THIM LARS ET AL) 6 September 2001 (2001-09-06) paragraphs [0023],[0041] ---	69,70
A	WO 01 87335 A (BRYANT HENRY UHLMAN ;HEIMAN MARK LOUIS (US); LILLY CO ELI (US)) 22 November 2001 (2001-11-22) page 13, line 25 -page 14, line 11 ---	1-76
A	NAKAZATO M ET AL: "A role for ghrelin in the central regulation of feeding" NATURE, vol. 409, no. 6817; 11 January 2001 (2001-01-11), pages 194-198, XP002268478 page 195, right column paragraph 2 ---	1-64, 69-76
A	WO 02 066056 A (NIELSEN KLAUS GREGORIUS ;KOEFOED PETER (DK); PHARMEXA AS (DK)) 29 August 2002 (2002-08-29) abstract page 3, line 28 -page 5, line 25 ---	1-64, 69-76
P,X	WO 02 070711 A (CROWE JAMES SCOTT ;ASHMAN CLAIRE (GB); GLAXO GROUP LTD (GB); ELLIS) 12 September 2002 (2002-09-12) page 2, line 14 -page 4, line 2 page 10, line 11 page 51; claim 1 page 53; claims 15-19 -----	1-70,73, 75

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 03/00592

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-48, 61, 65-68 all partly
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☒ Claims Nos.: 49-52, 73, 75 all partly
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Claims Nos.: 1-48, 61, 65-68 all partly

Claims 1-48 and 61 relate to methods of treatment of the human or animal body by surgery or by therapy or diagnostic methods practiced on the human or animal body (PCT Rule 39.1(iv)). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compositions, namely the induction of an immune response against ghrelin.

Claims 65-68 include in vivo testing, and do therefore relate to methods of treatment of the human or animal body by surgery or by therapy or diagnostic methods practiced on the human or animal body (PCT Rule 39.1(iv)). Nevertheless, a search has been executed for these claims. The search has been executed as if the claims relate to a method performed entirely in vitro.

Continuation of Box I.2

Claims Nos.: 49-52, 73, 75 all partly

Present claims 49-52, 73 and 75 relate to an extremely large number of possible compounds and uses of such compounds. The claims cover compounds wherein a ghrelin polypeptide is modified in any possible way which renders it immunogenic, thus including polypeptides modified for other purposes but nevertheless being immunogenic. Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts related to the compounds mentioned in claim 19 (SEQ ID NOs:1-5), the compounds used in the examples (SEQ ID NOs: 15-17) and the general idea of the invention: inducing an immune response against ghrelin, especially by active immunotherapy.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK 03/00592

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0153312	A	26-07-2001	US 6569662 B1	27-05-2003
			AU 2292401 A	31-07-2001
			AU 2591801 A	31-07-2001
			AU 2593601 A	31-07-2001
			AU 2595501 A	31-07-2001
			AU 2596501 A	31-07-2001
			AU 2598301 A	31-07-2001
			AU 2728401 A	31-07-2001
			AU 2734401 A	31-07-2001
			AU 2734801 A	31-07-2001
			AU 2738501 A	31-07-2001
			AU 3265701 A	31-07-2001
			CA 2395443 A1	26-07-2001
			CA 2395731 A1	26-07-2001
			CA 2395736 A1	26-07-2001
			CA 2395749 A1	26-07-2001
			CA 2395763 A1	26-07-2001
			CA 2395770 A1	26-07-2001
			CA 2402563 A1	26-07-2001
			EP 1242596 A1	25-09-2002
			EP 1240178 A2	18-09-2002
			EP 1242580 A1	25-09-2002
			EP 1242443 A1	25-09-2002
			EP 1250346 A2	23-10-2002
			EP 1254256 A2	06-11-2002
			EP 1248848 A1	16-10-2002
			WO 0153312 A1	26-07-2001
			WO 0153453 A2	26-07-2001
			WO 0153326 A1	26-07-2001
			WO 0153454 A2	26-07-2001
			WO 0153455 A2	26-07-2001
			WO 0153456 A2	26-07-2001
			WO 0153466 A1	26-07-2001
			WO 0152616 A2	26-07-2001
			WO 0153500 A1	26-07-2001
			WO 0153515 A1	26-07-2001
			WO 0153485 A1	26-07-2001
			US 2003104529 A1	05-06-2003
			US 2003219744 A1	27-11-2003
			US 2003211987 A1	13-11-2003
			US 2003224379 A1	04-12-2003
			US 6586390 B1	01-07-2003
			US 6465620 B1	15-10-2002
			US 2002146692 A1	10-10-2002
			US 6667391 B1	23-12-2003
			AU 5362001 A	30-10-2001
			CA 2406039 A1	25-10-2001
			EP 1276754 A2	22-01-2003
			WO 0179446 A2	25-10-2001
			US 2002197679 A1	26-12-2002
WO 0236792	A	10-05-2002	AU 1250502 A	15-05-2002
			CA 2427952 A1	10-05-2002
			EP 1334201 A2	13-08-2003
			WO 0236792 A2	10-05-2002
US 2001020012	A1	06-09-2001	AU 2832501 A	14-08-2001
			WO 0156592 A1	09-08-2001

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK 03/00592

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0187335	A	22-11-2001	AU 5905601 A	26-11-2001
			EP 1286697 A2	05-03-2003
			WO 0187335 A2	22-11-2001

WO 02066056	A	29-08-2002	US 2002187157 A1	12-12-2002
			AU 3362001 A	03-09-2001
			BR 0108566 A	19-11-2002
			CA 2400838 A1	30-08-2001
			CA 2440197 A1	29-08-2002
			WO 02066056 A2	29-08-2002
			EE 200200444 A	15-12-2003
			EP 1259251 A2	27-11-2002
			EP 1363664 A2	26-11-2003
			HU 0300067 A2	28-05-2003
			JP 2003523402 T	05-08-2003
			NO 20023961 A	20-08-2002
			NZ 521442 A	26-09-2003
			US 2002119162 A1	29-08-2002
			WO 03015812 A2	27-02-2003
			US 2003157117 A1	21-08-2003

WO 02070711	A	12-09-2002	CA 2439628 A1	12-09-2002
			CZ 20032373 A3	14-01-2004
			EP 1368477 A1	10-12-2003
			WO 02070711 A1	12-09-2002
			HU 0303372 A2	28-01-2004
			NO 20033882 A	31-10-2003
			US 2003194391 A1	16-10-2003
